



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification<sup>6</sup>:

(11) International Publication Number:

WO 95/06067

C07K 16/30, C12P 21/08, A61K 47/48, A1  
39/395, G01N 33/577, C07K 19/00

(43) International Publication Date:

2 March 1995 (02.03.95)

(21) International Application Number: PCT/GB94/01816

(22) International Filing Date: 19 August 1994 (19.08.94)

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(30) Priority Data:

9317423.3

21 August 1993 (21.08.93)

GB

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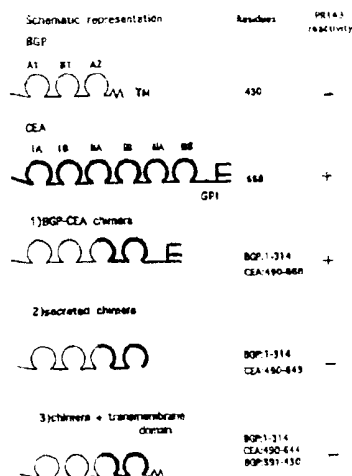
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(81) Designated States: AM, AT, AU, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, ES, FI, GB, GE, HU, JP, KE, KG, KP, KR, KZ, LK, LT, LU, LV, MD, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD, SE, SI, SK, TJ, TT, UA, US, UZ, VN, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG), ARIPO patent (KE, MW, SD).

## Published

With international search report.

(54) Title: MONOCLONAL ANTIBODIES FOR USE IN DIAGNOSIS AND TREATMENT OF COLORECTAL CANCER



## (57) Abstract

A molecule which (i) binds human membrane-bound carcinoembryonic antigen, (ii) binds a hybrid polypeptide consisting of residues 1 to 314 of human biliary glycoprotein joined (N-C) to residues 490 to C-terminus of human carcino embryonic antigen, but (iii) does not bind to human biliary glycoprotein excluding an intact mouse monoclonal antibody comprising an IgG group IIA heavy chain and a kappa group V light chain wherein the sequence of the V<sub>H</sub> chain is QVKLQQSGPELKKPGETVKISCKASGYTFTVFGMNNWVKQAPGKGLKWMGWIN-TKTGEATYVEEFKGRFAFSLETSATTA YLQINLNKNEDTAKYFCARWDFDYDYVEAMDYWGQGTTVTSS, or wherein the sequence of the V<sub>H</sub> chain is as given immediately above but the first amino acid residue of the V<sub>H</sub> CDR1 is glutamine and in either case the sequence of the V<sub>L</sub> chain is GDIVMTQSQRFMSTSVGDRSVTCKASQNVGTNVAWYQQKPGQSPKALITYSASYRSGVDPDRFTGSG-SGTDFTLTISNVQSEDLAEYFCHQYYTYPLFTFGSGTKLEMKR. Preferably the molecule is a monoclonal antibody.

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## MONOCLONAL ANTIBODIES FOR USE IN DIAGNOSIS AND TREATMENT OF COLORECTAL CANCER

The present invention relates to antibodies useful in diagnosing and treating colorectal cancer.

5

Antibodies are known that react with carcino-embryonic antigen (CEA), but they react with both membrane-associated CEA and soluble CEA and so are not especially useful in diagnosing colorectal cancer.

- 10 Monoclonal antibody PR1A3 was raised by fusion of NS1 (P3/NS1/I-Ag-4-1) myeloma cells with spleen cells from mice immunised with normal colorectal epithelium (Richman & Bodmer 1987). PR1A3 reacts strongly to both well and poorly differentiated colorectal carcinomas and has advantages over other colorectal epithelium-reactive antibodies since its
- 15 antigen appears fixed to the tumour and does not appear in the lymphatics or normal lymph nodes draining a tumour (Granowska *et al* 1989). PR1A3 reacted with 59/60 colorectal tumours (Richman & Bodmer 1987), whereas CEA reactive B72.3 reacted with only 75% (Salvatore *et al* 1989). Although there is some evidence for weak binding to normal cells
- 20 of the stomach, ileum, oesophagus, trachea and breast, *in vivo* studies have shown that the basement membrane prevents access by the antibody to these tissues (Granowska *et al* 1990).

PR1A3 has been distributed publicly, as immunoglobulin, although the

25 hybridoma has not been made available. The precise epitope to which PR1A3 binds has not previously been known.

The present invention seeks to provide further molecules, including monoclonal antibodies with the same or better specificity for colorectal

30 cancer as PR1A3. Such antibodies may be prepared by raising MAbs to

the newly discovered PRIA3 epitope which we have now found is part of the carcino-embryonic antigen (CEA), a tumour marker expressed in colorectal carcinomas.

- 5 A first aspect of the present invention provides a molecule which (i) binds membrane-bound human carcinoembryonic antigen (CEA), (ii) binds a hybrid polypeptide consisting of residues 1 to 314 of human biliary glycoprotein (BGP) joined (N-C) to residues 490 to C-terminus of intact human CEA, but (iii) does not bind to human BGP, but excluding an  
10 intact mouse monoclonal antibody comprising an IgG<sub>1</sub> group IIA heavy chain and a kappa group V light chain wherein the sequence of the V<sub>H</sub> chain is

QVKLQQSGPELKKPGETVKISCKASGYTFTVFGMNWVKQAPGKGLKWMGWINTKTGEATY  
VEEFKGRFAFSLETSATTAYLQINNKNEDTAKYFCARWDFDYVEAMDYWGQGTTVTVS

- 15 s (SEQ ID No 1)

, or wherein the sequence of the V<sub>H</sub> chain is as given immediately above but the first amino acid residue of the V<sub>H</sub> CDR1 is glutamine

- 20 and in either case the sequence of the V<sub>L</sub> chain is

GDIVMTQSQRFMSTSVGDRVSVTCASQNVGTNVAWYQQKPGQSPKALIYSASYRYSQV  
DRFTGSGSGTDFTLTISNVQSEDLAEYFCHQYYTYPLFTFGSGTKLEMKR (SEQ ID No 2)

The sequence of the V<sub>H</sub> chain can also be written as:

- 25 Gln Val Lys Leu Gln Gln Ser Gly Pro Glu Leu Lys Lys Pro Gly Glu  
Thr Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Val Phe  
Gly Met Asn Trp Val Lys Gln Ala Pro Gly Lys Gly Leu Lys Trp Met  
Gly Trp Ile Asn Thr Lys Thr Gly Glu Ala Thr Tyr Val Glu Glu Phe  
30 Lys Gly Arg Phe Ala Phe Ser Leu Glu Thr Ser Ala Thr Thr Ala Tyr  
Leu Gln Ile Asn Asn Leu Lys Asn Glu Asp Thr Ala Lys Tyr Phe Cys  
Ala Arg Trp Asp Phe Tyr Asp Tyr Val Glu Ala Met Asp Tyr Trp Gly  
Gln Gly Thr Thr Val Thr Val Ser Ser

The sequence of the V<sub>L</sub> chain can also be written as:

5 Gly Asp Ile Val Met Thr Gln Ser Gln Arg Phe Met Ser Thr Ser Val  
 Gly Asp Arg Val Ser Val Thr Cys Lys Ala Ser Gln Asn Val Gly Thr  
 Asn Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Ala Leu  
 Ile Tyr Ser Ala Ser Tyr Arg Tyr Ser Gly Val Pro Asp Arg Phe Thr  
 Gly Ser Gly Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Asn Val Gln  
 Ser Glu Asp Leu Ala Glu Tyr Phe Cys His Gln Tyr Tyr Thr Tyr Pro  
 Leu Phe Thr Phe Gly Ser Gly Thr Lys Leu Glu Met Lys Arg

10

The first amino acid residue of the V<sub>H</sub> CDR1 is a position 31 in the V<sub>H</sub> sequence given.

It is preferred if the molecule is an antibody.

15

The hybrid polypeptide consisting of the N-A1-B1-(N-terminal half of A2) domains of BGP joined (N-C) to the (C-terminal half of A3)-B3-GPI domains of human CEA is described in detail in Example 1 and shown diagrammatically as chimaera 1 in Figure 8. It consists of residues 1 to  
 20 314 of BGP fused to residues 490 - C-terminus of CEA in a N-C fashion.  
 The C-terminus of intact CEA is residue 668.

By "membrane-bound" we mean CEA as found in a colon carcinoma cell, for example the HT-29 cell line, a moderately well-differentiated grade II  
 25 human colon adenocarcinoma cell line available from the American Type Culture Collection, 12301 Parklawn Drive, Rockville, Maryland 20852, USA under catalogue number ATCC HTB 38.

By "binding" we mean that, when assessed by indirect  
 30 immunofluorescence of acetone-fixed CEA-positive cell monolayers grown in cover slips, a positive signal is seen following incubation with a fluorescence conjugate anti-mouse (or human) IgG antibody. Example 7

describes a method of acetone fixation of cells and determination of binding.

That an antibody heavy chain is IgG<sub>1</sub> can be determined by reaction with  
5 antisera specific for the isotype sera in immunodiffusion gels (Ouchterlony  
technique) or by enzyme-linked immunosorbent assays (ELISA).  
Monoclonal antibodies which react against, and are diagnostic for, mouse  
IgG heavy chain are commercially available, for example the rat  
monoclonal antibody clone name LO-MG1-2 available from Serotec, 22  
10 Bankside, Station Approach, Kidlington, Oxford OX5 1JE, UK, and has  
an avidity of  $9 \times 10^8 \text{ M}^{-1}$ .

That an antibody light chain is kappa can be determined by reaction with  
specific antisera in immunodiffusion gels and by ELISA. Monoclonal  
15 antibodies which react against, and are diagnostic for, mouse kappa light  
chain are commercially available, for example the rat monoclonal antibody  
clone name MRC OX-20 available from Serotec.

IgG group IIA and kappa group V refer to sub-types of the V-regions and  
20 are defined by the sequence of the V-region frameworks as described by  
Kabat *et al* (1991) *Sequence of Proteins of Immunological Interest*, fifth  
edition, US Department of Health and Human Services, NIH Publication  
No 91-3242 incorporated herein by reference.

25 It is preferred if the molecule does not bind substantially to other naturally  
occurring human proteins that are present in the human body and whose  
location is in the bowel. Such proteins include collagen and serum  
albumin.

30 It is preferred if the molecule does not bind to N-A1-Fc, N-A1-B1-Fc or

N-A1-B1-A2-Fc where in A1, B1 and A2 are domains of CEA and Fc is the Fc portion of immunoglobulin.

It is further preferred if the molecule does not bind a B3 hybrid wherein  
5 the GPI anchor is removed or wherein the GPI anchor is replaced with a BGP transmembrane segment.

When the molecule is an antibody it is preferred if it comprises a human framework region and at least the complementarity determining regions of  
10 the V<sub>H</sub> chain and V<sub>L</sub> chain as defined in Claim 1 wherein for the V<sub>H</sub> chain CDR1 is VFGMN (SEQ ID No 3), CDR2 is WINTKTGEATYVEEFKG (SEQ ID No 4) and CDR3 is WDFYDYVEAMDY (SEQ ID No 5) and for the V<sub>L</sub> chain CDR1 is KASQNVGTNVA (SEQ ID No 6), CDR2 is SASYRYS (SEQ ID No 7) and CDR3 is HQYYTYPLFT (SEQ ID No 8).

15 PR1A3 is a mouse monoclonal antibody comprising an IgG<sub>1</sub> group IIA heavy chain and a kappa group V light chain wherein the sequence of the V<sub>H</sub> chain is as stated above in the exclusion from the first aspect of the invention or wherein the first amino acid residue of the V<sub>H</sub> CDR1 is  
20 glutamine and the sequence of the V<sub>L</sub> chain is as stated above in the exclusion from the first aspect of the invention.

CEA is a member of the immunoglobulin super-gene family (reviewed in Thompson & Zimmermann 1988; Thompson *et al* 1991). CEA has a  
25 domain structure of N-A1-B1-A2-B2-A3-B3-GPI where GPI is a glycoposphatidylinositol membrane anchor. A significant degree of sequence homology exists between the domains of CEA and with other members of the family such as NCA.

30 Biliary glycoprotein (BGP) is also a member of the immunoglobulin gene

super-family and has a domain structure of N-A1-B1-A2-TM, where TM is a transmembrane domain, but the domains A1, B1 and A2 of BGP are not identical to those named A1, B1 and A2 in CEA.

- 5 By "antibody", we include monoclonal and polyclonal antibodies and we include antibody fragments which bind specifically but reversibly to (i) human CEA, (ii) a hybrid polypeptide consisting of residues 1 to 314 of human BGP joined (N-C) to residues 490 to C-terminus of human CEA but (iii) do not bind to human BGP excluding an intact mouse monoclonal  
10 antibody comprising an IgG1 group IIA heavy chain and a kappa group V light chain wherein the sequence of the V<sub>H</sub> chain is (as defined in Figure 1) or wherein the first amino acid residue of the V<sub>H</sub> CDR1 is glutamine and the sequence of the V<sub>L</sub> chain (is as defined in Figure 2).
- 15 It is preferred if the antibody or antibody fragment is derived from a monoclonal antibody.

Monoclonal antibodies may be prepared generally by the techniques of Zola, H. (1988) (*"Monoclonal Antibodies - A manual of techniques"* CRC  
20 Press) which is incorporated herein by reference. Antibody fragments such as Fab, (Fab)<sub>2</sub>, Fv, scFv or dAb fragments may be prepared therefrom in known ways. The antibodies may be humanized in known ways for example, by inserting the CDR regions of mouse antibodies into the framework of human antibodies. Antibody-like molecules may be  
25 prepared using the recombinant DNA techniques of WO 84/03712. The region specific for the protein may be expressed as part of a bacteriophage, using the technique of McCafferty *et al* (1990) *Nature* **348**, 552-554.

30 Antibody-like molecules of the invention may be selected from phage



display libraries using the methods described in Griffiths *et al* (1993) *EMBO J.* **12**, 725-734 where CEA or hybrid proteins expressed in cells are immobilized and used to select phages. Also, appropriate cells grown in monolayers and either fixed with formaldehyde or glutaraldehyde or  
5 unfixed can be used to bind phages. Irrelevant phages are washed away and bound phages recovered by disrupting their binding to the CEA or hybrid protein and reamplifying in bacteria. This selection and amplification process is done several times to enrich the phage population for those molecules which are the antibody-like molecules of the  
10 invention.

We also include peptides selected from random peptide libraries in a similar way to those from phage display libraries in the antibody-like molecules of the invention.

15

The variable heavy ( $V_H$ ) and variable light ( $V_L$ ) domains of the antibody are involved in antigen recognition, a fact first recognised by early protease digestion experiments. Further confirmation was found by "humanisation" of rodent antibodies. Variable domains of rodent origin  
20 may be fused to constant domains of human origin such that the resultant antibody retains the antigenic specificity of the rodent parent antibody (Morrison *et al* (1984) *Proc. Nat. Acad. Sci. USA* **81**, 6851-6855) or "CDR grafting" can be used to humanise rodent antibodies. Additionally or alternatively, recombinant monoclonal antibodies may be "primatised",  
25 ie antibodies formed in which the variable region of the heavy and light chains, or parts thereof, and the constant regions are derived from two different primate species, preferably the variable regions of the antibody from the macaque monkey, and the constant regions from human. The advantages of such antibodies include high homology to human  
30 immunoglobulin, presence of human effector functions, reduced

immunogenicity and longer serum half-life (Newman *et al* (1992) *Biotechnology* **10**, 1455).

That antigenic specificity is conferred by variable domains and is  
5 independent of the constant domains is known from experiments involving  
the bacterial expression of antibody fragments, all containing one or more  
variable domains. These molecules include Fab-like molecules (Better *et al* (1988) *Science* **240**, 1041); Fv molecules (Skerra *et al* (1988) *Science*  
**240**, 1038); single-chain Fv (ScFv) molecules where the  $V_H$  and  $V_L$   
10 partner domains are linked via a flexible oligopeptide (Bird *et al* (1988) *Science* **242**, 423; Huston *et al* (1988) *Proc. Natl. Acad. Sci. USA* **85**,  
5879) and single domain antibodies (dAbs) comprising isolated V domains  
(Ward *et al* (1989) *Nature* **341**, 544). A general review of the techniques  
involved in the synthesis of antibody fragments which retain their specific  
15 binding sites is to be found in Winter & Milstein (1991) *Nature* **349**, 293-  
299.

By "ScFv molecules" we mean molecules wherein the  $V_H$  and  $V_L$  partner  
domains are linked via a flexible oligopeptide.

20

In certain circumstances there are advantages of using antibody fragments,  
rather than whole antibodies. The smaller size of the fragments allows for  
rapid clearance, and may lead to improved tumour to non-tumour ratios.  
Fab, Fv, ScFv and dAb antibody fragments can all be expressed in and  
25 secreted from *E. coli*, thus allowing the facile production of large amounts  
of the said fragments.

Whole antibodies, and  $F(ab')_2$  fragments are "bivalent". By "bivalent"  
we mean that the said antibodies and  $F(ab')_2$  fragments have two antigen  
30 combining sites. In contrast, Fab, Fv, ScFv and dAb fragments are

monovalent, having only one antigen combining site.

The art of "antibody engineering" is advancing rapidly, as is described in Tan, L.K. and Morrison, S.L. (1988) *Adv. Drug Deliv. Rev.* **2**: 129-142,  
5 Williams, G. (1988) *Tibtech* **6**: 36-42 and Neuberger, M.S. *et al* (1988) *8th International Biotechnology Symposium Part 2*, 792-799 (all of which are incorporated herein by reference), and is well suited to preparing antibody-like molecules derived from the antibodies of the invention.

- 10 The antibodies may be used for a variety of purposes relating to the study or isolation and purification of the antigen to which they specifically bind and the imaging and treatment of cells exhibiting the antigen. In other embodiments, the antibody of the invention is coupled to a scintigraphic radiolabel, a cytotoxic compound or radioisotope, an enzyme for  
15 converting a non-toxic prodrug into a cytotoxic drug, a compound for activating the immune system in order to target the resulting conjugate to a colon tumour, or a cell-stimulating compound. Such conjugates have a "binding portion", which consists of the antibody of the invention, and a "functional portion", which consists of the radiolabel, toxin or enzyme  
20 etc.

The antibody may alternatively be used alone in order simply to block the activity of the CEA antigen, particularly by physically interfering with its binding of another compound.

25

- The binding portion and the functional portion of the conjugate (if also a peptide or polypeptide) may be linked together by any of the conventional ways of cross-linking polypeptides, such as those generally described in O'Sullivan *et al* (1979) *Anal. Biochem.* **100**, 100-108. For example, one  
30 portion may be enriched with thiol groups and the other portion reacted

with a bifunctional agent capable of reacting with those thiol groups, for example the N-hydroxysuccinimide ester of iodoacetic acid (NHIA) or N-succinimidyl-3-(2-pyridyldithio)propionate (SPDP). Amide and thioether bonds, for example achieved with *m*-maleimidobenzoyl-N-hydroxysuccinimide ester, are generally more stable *in vivo* than disulphide bonds.

Alternatively, if the binding portion contains carbohydrates, such as would be the case for an antibody or some antibody fragments, the functional portion may be linked via the carbohydrate portion using the linking technology in EP 0 088 695.

The functional portion of the conjugate may be an enzyme for converting a non-toxic prodrug into a toxic drug, for example the conjugates of Bagshawe and his colleagues (Bagshawe (1987) *Br. J. Cancer* **56**, 531; Bagshawe *et al* (1988) *Br. J. Cancer* **58**, 700; WO 88/07378) or cyanide-releasing systems (WO 91/11201).

It may not be necessary for the whole enzyme to be present in the conjugate but, of course, the catalytic portion must be present. So-called "abzymes" may be used, where a monoclonal antibody is raised to a compound involved in the reaction one wishes to catalyse, usually the reactive intermediate state. The resulting antibody can then function as an enzyme for the reaction.

25

The conjugate may be purified by size exclusion or affinity chromatography, and tested for dual biological activities. The antigen immunoreactivity may be measured using an enzyme-linked immunosorbent assay (ELISA) with immobilised antigen and in a live cell radio-immunoassay. An enzyme assay may be used for  $\beta$ -glucosidase

30

using a substrate which changes in absorbance when the glucose residues are hydrolysed, such as *o*NPG (*o*-nitrophenyl- $\beta$ -D-glucopyranoside), liberating 2-nitrophenol which is measured spectrophotometrically at 405 nm.

5

Stability of the conjugate may be tested *in vitro* initially by incubating at 37°C in serum, followed by size exclusion FPLC analysis. Stability *in vivo* can be tested in the same way in mice by analysing the serum at various times after injection of the conjugate. In addition, it is possible  
10 to radiolabel the antibody with  $^{125}\text{I}$ , and the enzyme with  $^{131}\text{I}$  before conjugation, and to determine the biodistribution of the conjugate, free antibody and free enzyme in mice.

Alternatively, the conjugate may be produced as a fusion compound by  
15 recombinant DNA techniques whereby a length of DNA comprises respective regions encoding the two portions of the conjugate either adjacent one another or separated by a region encoding a linker peptide which does not destroy the desired properties of the conjugate.

20 Conceivably, the two functional portions of the compound may overlap wholly or partly. The DNA is then expressed in a suitable host in known ways.

The conjugates may be administered in any suitable way, usually  
25 parenterally, for example intravenously or intraperitoneally, in standard sterile, non-pyrogenic formulations of diluents and carriers, for example isotonic saline (when administered intravenously). Once the conjugate has bound to the target cells and been cleared from the bloodstream (if necessary), which typically takes a day or so, the pro-drug is  
30 administered, usually as a single infused dose, or the tumour is imaged.

If needed, because the conjugate may be immunogenic, cyclosporin or some other immunosuppressant can be administered to provide a longer period for treatment but usually this will not be necessary.

- 5 The timing between administrations of conjugate and pro-drug may be optimised in a non-inventive way since tumour/normal tissue ratios of conjugate (at least following intravenous delivery) are highest after about 4-6 days, whereas at this time the absolute amount of conjugate bound to the tumour, in terms of percent of injected dose per gram, is lower than  
10 at earlier times.

Therefore, the optimum interval between administration of the conjugate and the pro-drug will be a compromise between peak tumour concentration of enzyme and the best distribution ratio between tumour and normal  
15 tissues. The dosage of the conjugate will be chosen by the physician according to the usual criteria. At least in the case of methods employing a targeted enzyme such as  $\beta$ -glucosidase and intravenous amygdalin as the toxic pro-drug, 1 to 50 daily doses of 0.1 to 10.0 grams per square metre of body surface area, preferably 1.0-5.0 g/m<sup>2</sup> are likely to be appropriate.  
20 For oral therapy, three doses per day of 0.05 to 10.0g, preferably 1.0-5.0g, for one to fifty days may be appropriate. The dosage of any conjugate will similarly be chosen according to normal criteria, particularly with reference to the type, stage and location of the tumour and the weight of the patient. The duration of treatment will depend in  
25 part upon the rapidity and extent of any immune reaction to the conjugate.

The functional portion of the conjugate, when the conjugate is used for diagnosis, usually comprises and may consist of a radioactive atom for scintigraphic studies, for example technetium 99m (<sup>99m</sup>Tc) or iodine-123  
30 (<sup>123</sup>I), or a spin label for nuclear magnetic resonance (nmr) imaging (also

known as magnetic resonance imaging, mri), such as iodine-123 again, iodine-131, indium-111, fluorine-19, carbon-13, nitrogen-15, oxygen-17, gadolinium, manganese or iron.

- 5 When used in a compound for selective destruction of the tumour, the functional portion may comprise a highly radioactive atom, such as iodine-131, rhenium-186, rhenium-188, yttrium-90 or lead-212, which emits enough energy to destroy neighbouring cells, or a cytotoxic chemical compound such as methotrexate, adriamycin, vinca alkaloids (vincristine,  
10 vinblastine, etoposide), daunorubicin or other intercalating agents.

The radio- or other labels may be incorporated in the conjugate in known ways. For example, the peptide may be biosynthesized or may be synthesized by chemical amino acid synthesis using suitable amino acid  
15 precursors involving, for example, fluorine-19 in place of hydrogen. Labels such as  $^{99m}\text{Tc}$ ,  $^{123}\text{I}$ ,  $^{186}\text{Rh}$ ,  $^{188}\text{Rh}$  and  $^{111}\text{In}$  can be attached via a cysteine residue in the peptide. Yttrium-90 can be attached via a lysine residue. The IODOGEN method (Fraker *et al* (1978) *Biochem. Biophys. Res. Commun.* **80**: 49-57 can be used to incorporate iodine-123.  
20 "Monoclonal Antibodies in Immunoscintigraphy" (Chatal, CRC Press 1989) describes other methods in detail.

Thus, a second aspect of the invention provides a molecule, preferably an antibody, as defined in the first aspect of the invention for use in  
25 medicine.

A third aspect of the invention provides a use of a molecule, preferably antibody, as defined by the first aspect of the invention in the manufacture of a medicament for use in the diagnosis or treatment of colorectal  
30 carcinoma.

A fourth aspect of the invention provides a process for making a monospecific antibody, the process comprising screening a pool of antibodies to select those monospecific antibodies which bind (i) human membrane-based CEA, (ii) bind a hybrid polypeptide consisting of residues 1 to 314 of human BGP joined (N-C) to residues 490 to C-terminus of human CEA, but (iii) do not bind to human BGP.

It is preferred if the monospecific antibody is a monoclonal antibody and the pool of antibodies is a pool of monoclonal antibodies. It is further preferred if the antibodies within the pool comprise antibodies produced by recombinant DNA methods.

In the preferred embodiment the screening steps are for antibodies that:

(1) Bind to human tumour cells such as colorectal carcinoma cells (Richman & Bodmer (1987) *Int. J. Cancer* **39**, 317-328) and the human gastric carcinoma cell line MKN 45 (Kojama *et al* (1990) *Jpn. J. Cancer* **81**, 967-970). Binding is detected in indirect immunofluorescent assays where the cells are fixed to microscope slides or cover slips, for example with acetone, and antibody binding detected by a second fluorescently-labelled anti-species antibody, for example a FITC labelled anti-mouse IgG if the first antibody is a mouse IgG. Alternatively antibody binding to cells in suspension could be measured; and antibody binding could be detected by radioactively-labelled second antibody, for example by <sup>125</sup>I-labelled anti-mouse IgG.

(2) Bind to cells transfected with and expressing the human CEA. For example, these could be the simian virus 40-transformed monkey fibroblast line COS-7 transfected by electroporation with a CEA cDNA (Beauchemin *et al* (1987) *Mol. Cell. Biol.* **7**, 3221-3230) in the vector



- pCDM8 (Invitrogen); Chinese hamster ovary cells (CHO) transfected by electroporation with a CEA cDNA in the dexamethasone inducible vector pMAMneo (Clontech): a cosmid clone for CEA (Willcocks, T.C. & Craig, I.W. (1990) *Genomics* **8**, 492-500) co-transfected into the mouse colorectal carcinoma cell line CMT93 by lipofection with the plasmid pSVneo2; CHO cells transfected with a yeast artificial chromosome or YAC containing the CEA gene cluster, eg ICRFy9000C02400 from the q13.1-q13.3 region of the long arm of chromosome 19 and modified to include a neomycin resistance (*neo<sup>R</sup>*) gene by homologous recombination with the plasmid vector pRAN4 (Ragoussis *et al* (1992) *Nucleic Acids Res.* **20**, 3135-3138) with the right hand vector arm of pYAC4, transfection could be by yeast spheroplast cell fusion (Burgers, P. & Percival, K. (1987) *Anal. Biochem* **163**, 391-397).
- 15 (3) Bind to cells transfected with and expressing the hybrid gene BGP-CEAB3-GP1, for example COS-7 cells transfected by electroporation with the plasmid pCDM8 carrying the hybrid gene. Electroporation is described in Example 6.
- 20 (4) Do not bind to cells expressing BGP but not expressing CEA, for example COS-7 cells transfected with the plasmid pCDM8 carrying the cDNA for BGP.
- (5) Do not bind to cells expressing NCA but not expressing CEA, for  
25 example COS-7 cells transfected with the plasmid pCDM8 carrying the cDNA for NCA (Hefta *et al* (1990) *Cancer Res.* **50**, 2397-2403).
- (6) Do not bind to cells expressing the hybrid BGP-CEAB3 but without the GPI anchor, these cells could be transfected COS-7 cells  
30 transfected with the plasmid pCDM8 carrying the hybrid gene for BGP-

CEAB3 where a stop codon is introduced into the CEAB3 sequence at the beginning of the position of the hydrophobic tail which is normally processed off and replaced by a GPI anchor. PCR can be used to introduce such a stop codon.

5

(7) Do not bind to a cell expressing BGP-CEAB3-BGP TM, for example COS-7 transected with pCDM8 carrying the hybrid gene where the transmembrane domain of BGP was added to the B3 domain of CEA in place of the processed hydrophobic segment of CEA.

10

A useful control antibody that does not bind CEA is one that, for example, recognises the T-cell marker CD4. Suitable anti-CD4 antibodies are available from the ATCC, for example OKT4 (anti-human helper T cell subset; ATCC CRL 8002).

15

Selecting the antibodies of the invention can be done using the above steps in any permutation.

20

It is preferred if primary screening is done on a CEA-expressing cell line which can be a human tumour cell line or a transfectoma expressing CEA from a cDNA or cosmid.

25

It is preferred if secondary screening is done on cell lines transfected with the above mentioned genes and hybrid genes.

30

NCA is non-specific cross reacting antigen and comprises N, A1 and B1 domains and a GPI anchor (see Thomson & Zimmerman (1988) *Tumour Biol.* **9**, 63-83 and Thomson *et al* (1991) *J. Clin. Lab. Analysis* **5**, 344-366 for reviews).

Suitable parent cell lines for expression include COS cells and CHO cells which do not express CEA.

The invention will now be described in detail with reference to the following Examples and Figures wherein:

Figure 1 shows the deduced amino acid sequence for the V<sub>H</sub> chain of murine monoclonal antibody (murine heavy; SEQ ID No 1), its comparison with the V<sub>H</sub> sequence of the human antibody RF-TS3'CL used to provide the framework sequences for humanisation (RF-TS3 backbone; SEQ ID No 27), and the humanised sequence created (humanised heavy; SEQ ID No 28). The sequence of RF-TS3'CL is disclosed in Pascual *et al* (1990) *J. Clin. Invest.* **86**, 1320-1328 incorporated herein by reference.

Figure 2 shows the deduced amino acid sequence for the V<sub>L</sub> chain of murine monoclonal antibody (murine kappa; SEQ ID No 2), its comparison with the V<sub>L</sub> sequence of the human antibody REI used to provide the framework sequences for humanisation (REI backbone; SEQ ID No 29), and the humanised sequence created (humanised kappa; SEQ ID No 30).

Figure 3 shows the amino acid sequence comparison between CEA and NCA-50. Corresponding domains are grouped together. In each case, dots indicate identity to the amino acids of the CEA domains shown in the top line of each group. Dashes indicate amino acid deletions in comparison with CEA. Potential N-glycosylation positions are underlined.

Figure 4 shows the cDNA sequence (SEQ ID No 31) and deduced amino acid sequence (SEQ ID No 32) of BGP.

Figure 5 shows the structures of GPI.

Figure 6 shows the construction of a humanised heavy chain. FR indicates framework regions; CDR indicates complementarity determining regions; = indicates double stranded DNA encoding humanised heavy chain; and → indicates synthetic oligonucleotides, showing direction 5'-3', used as primers for overlapping PCR.

Figure 7 shows the construction of a humanised light chain. FR indicates framework regions; CDR indicates complementarity determining regions; = indicates double stranded DNA encoding humanised heavy chain; and → indicates synthetic oligonucleotides, showing direction 5'-3', used as primers for overlapping PCR.

Figure 8 shows the BGP-CEA chimaeric constructs.

Figure 9 shows a model of the V-domain of humanised antibody. The positions of the complementarity determining regions (CDRs) 1 to 3 of the light (L) and heavy (H) chains are shown. The two glutamic acid residues implicated in antigen recognition E(H:106) - position 106 of the heavy chain, and E(H:57) - position 57 of the heavy chain are marked.

Figure 10 shows a model of the B3 domain of CEA. The positions of the lysine and arginine residues are marked. *In vitro* mutagenesis of KG36 (lysine at position 636) and R594 (arginine position 594) destroys PR1A3 binding to the antigen.

Figure 11 shows the cDNA sequence (SEQ ID No 33) and deduced amino acid sequence (SEQ ID No 34) of NCA.

Figure 12 shows the cDNA sequence (SEQ ID No 35) and deduced amino acid sequence (SEQ ID No 36) of CEA.

Figure 13 shows the cDNA sequence (SEQ ID No 37) and deduced amino acid sequence (SEQ ID No 38) of the PR1A3 kappa light chain.

Figure 14 shows the cDNA sequence (SEQ ID No 39) and deduced amino acid sequence (SEQ ID No 40) of the PR1A3 heavy chain.

10 **Example 1: Identification of the epitope recognised by PR1A3**

YAC (yeast artificial chromosome) and cosmid studies have mapped the gene encoding the PR1A3 antigen to the chromosomal region in which the CEA gene is located and, like CEA, the PR1A3 epitope was shown to be up-regulated by  $\gamma$ -interferon. Transfection of a cDNA for CEA into a variety of cells gave the appearance of the PR1A3 epitope on these cells, thus indicating that the monoclonal antibody PR1A3 recognises an epitope on CEA.

20 Domains of CEA were expressed in COS cells as fusions to the Fc portion of immunoglobulin as N-A1-Fc, N-A1-B1-Fc and N-A1-B1-A2-Fc. None of these constructs produced protein which reacted with PR1A3, therefore the epitope is not located in the N-A1-B1-A2 region.

25 Hybrid constructs of BGP and CEA were made such that the (C-terminal half of A3)-B3-GPI domains of CEA were fused to the N-A1-B1-(N-terminal half of A2) domains of BGP. Amino acid sequences for CEA and BGP are shown in Figures 3 and 4. The hybrid construct number 1 contained BGP up to cysteine 314 and from glutamic acid 490 to the C-terminus of CEA (see Figure 8). The hybrid construct was expressed in

COS cells from the expression plasmid pCDM8. When analysed in immunofluorescence assays the transfected COS cells gave a positive signal with both the mouse PR1A3 antibody and a human/mouse chimaeric antibody (see below). This confirms that the PR1A3 epitope is in the region of the B3-GPI region. The plasmid pCDM8 is described in Seed & Aruffo (1987) *Proc. Natl. Acad. Sci. USA* **84**, 3365-3369.

A stop codon was inserted into the hybrid construct number 1 such that no GPI anchor was added to the protein. The CEA portion should no longer be membrane bound but soluble and secreted. The stop codon was inserted at a position equivalent to residue 644 of CEA and a protein of (N-A1-B1-(N-terminal half of A2)) BGP - ((C-terminal half of A3)-B3) CEA was formed (construct number 2; Figure 8). Transfection of this construct into COS cells using the vector pCDM8 gave cells which were positive in immunofluorescent studies with an antibody, 3B10, which cross-reacts with BGP, but negative for PR1A3. This confirms expression of the hybrid protein but that the PR1A3 epitope is absent when the hybrid is not membrane bound.

Chimaeric constructs 3a and 3b were made and the structures are as indicated in Figure 8.

#### PCR methods used in making expression constructs

For cloning BGP the following primers were used:

5' + *Hind*III site: CTCAAGCTTATGGGGCACCTC  
(SEQ ID No 9)

3' + *Xba*I site: GGTCTAGACTATGAAGTTGGTTG

(SEQ ID No 10)

For cloning CEA the following primers were used:

5' + *Hind*III site: CTCAAGCTTATGGAGTCTCCC  
(SEQ ID No 11)

5 3' + *Xba*I site: GGTCTAGACTATATCAGAGCAAC  
(SEQ ID No 12)

For chimaera 1 BGP and CEA fragments were amplified by PCR from parent molecules. Products were cut with *Cl*aI and ligated. 5' and 3' ends of the annealed product were cut with *Hind*III and *Xba*I for ligation into the *Hind*III-*Xba*I site of pCDM8 vector for transient expression in COS-7 cells.

The following primers were used:

15 5' + *Hind*III site: CTCAAGCTTATGGGGCACCTC  
(SEQ ID No 9)

3' B1 loop + *Cl*aI site: GGATCGATGCAGGTCAGGTT  
(SEQ ID No 13)

20 5' IIIA loop + *Cl*aI site: CTCATCGATGAACCTGAGGCT  
(SEQ ID No 14)

3' + *Xba*I site: GGTCTAGACTATATCAGAGCAAC  
(SEQ ID No 15)

25 Chimaera 2 was amplified from chimaera 1 and cut with *Hind*III and *Xba*I for ligation into pCDM8.

The following primers were used:

5' + *Hind*III site: CTCAAGCTTATGGGGCACCTC  
(SEQ ID No 9)

3' + STOP + *Xba*I site: GGTCTAGACTAAGATGCAGAGAC  
(SEQ ID No 16)

5

For making chimaeras 3a and 3b 5' portion of the molecule was amplified from chimaera 1 template using BGP 5' primer and antisense overlapping primer complementary to the required join. The BGP transmembrane domain was amplified from BGP using sense overlapping primer and BGP

10 3' primer.

To assemble the annealed molecule, the two complementary fragments were subjected to 18 PCR cycles to allow formation of "primer-dimer" before addition of BGP 5' and 3' outside primers for 12 further PCR  
15 cycles.

The following primers were used for construct 3a:

5' + *Hind*III site: CTCAAGCTTATGGGGCACCTC  
20 (SEQ ID No 9)

Overlapping primers: TCTGCATCTGGACTCTCACCTGGGGCC  
(sense) (SEQ ID No 17)  
GGCCCCAGGTGAGAGTCCAGATGCAGA  
(antisense) (SEQ ID No 18)

25 3' + *Xba*I site: GGTCTAGACTATGAAGTTGGTTG  
(SEQ ID No 10)

The following primers were used for construct 3b:



5' + *Hind*III site: CTCAAGCTTATGGGGCACCTC  
(SEQ ID No 9)

Overlapping primers: ACAGTCTCTGCACAAGAAAATGGC  
(sense) (SEQ ID No 19)

5 GCCATTTTCTTGTGCAGAGACTGT  
(antisense) (SEQ ID No 20)

3' + *Xba*I site: GGTCTAGACTATGAAGTTGGTTG  
(SEQ ID No 10)

- 10 Anchor structures are reviewed in Ferguson (1992) and a generic structure for a mammalian GPI is shown in Figure 5. Studies with CEA released from MKN45 cells by incubation with a phospholipase, which cleaves the lipid tail from GPI anchors to give a soluble product, produces CEA which contains the PR1A3 epitope. When examined by SDS PAGE and
- 15 western blotting a weak signal is given if this antigen is boiled in 2% SDS sample buffer with reducing agent dithiothreitol to break disulphide bridges. When the antigen is examined in the same way, but the reducing agent omitted to retain the disulphide bridges intact, a strong signal is given. This suggests the epitope is at least partly conformational.
- 20 Furthermore, NCA is related to CEA, with a high degree of sequence homology, and has a GPI anchor, but does not react with PR1A3. Therefore the GPI is unlikely to be sufficient for the epitope.

### **Example 2: Molecular modelling and *in vitro* mutagenesis**

25

Molecular models of the antibody PR1A3 demonstrate the presence of two unusual unpaired negative charges in the CDR region of the antibody. These charges may indicate the presence of complementary charges in the epitope recognised by the antibody (see Figures 9 and 10).

30

Analysis of the B3 domain of CEA and comparison with another family member, NCA, indicated that there were three residues carrying positive charges which could play an important role in the antibody antigen interaction. The residues were, lysines at positions 610 and 636 and  
5 arginine at position 514 in the CEA B3 domain. In order to assess the role of the individual charges in the epitope recognised by the antibody PR1A3, these residues were changed from lysine or arginine, to alanine. It is possible to alter these amino acids by changing the sequence of the DNA. The polymerase chain reaction may be used to introduce point  
10 mutations which are incorporated into one of the amplification primers. The fragment is then blunt-ended with Klenow fragment or digested with restriction endonucleases and ligated into the appropriate vector to allow the product to be sequenced. Alternatively, to introduce a mutation into the middle of a sequence, two fragments encompassing the mutation are  
15 annealed with each other and extended by mutually primed synthesis. The fragment may then be digested as before and ligated into an appropriate vector to be sequenced.

PCR may also be used to incorporate a phosphorylated oligonucleotide  
20 during amplification with *Taq* polymerase and *Taq* ligase (Michel, *BioTechniques* 16(3), 410-412).

Mutations may also be introduced by construction of a totally synthetic gene or portion of the gene.

25

The method which we used to introduce changes into the sequence was oligonucleotide directed mutagenesis by the method of Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82, 488-492).

30 The repetitive nature of CEA meant that in order to carry out the

mutagenesis the B3 domain and downstream sequences of CEA had to be isolated from similar upstream sequences. This region was amplified with primers to introduce a *Cl*I site 5' and an *X*baI site 3' and the fragment was cloned into pBluescriptII KS-. The plasmid was transformed into a *dur*<sup>-</sup>*ung*<sup>-</sup> F' strain of *E. coli* (CJ236) which will produce plasmid with a number of uracil residues in place of thymine. Single stranded template was produced by superinfection with a helper phage M13 K07. A phosphorylated oligonucleotide containing the mutant sequence is annealed to the template and extended in the presence of T4 DNA polymerase and ligase to produce a double-stranded circular molecule. Introduction of this heteroduplex molecule into a wild-type (*dur*<sup>+</sup>*ung*<sup>+</sup>) strain resulted in degradation of the uracil containing wild-type strand and replication of the mutant strand. Colonies were isolated and the DNA sequenced to ensure that the mutant genotype was present.

### Mutations

WILD-TYPE SEQUENCE ATC GCC AAA ATC ACG (SEQ ID No 21)

K1 MUTANT OLIGO ATC GCC GCA ATC ACG (SEQ ID No 22)

WILD-TYPE SEQUENCE ATA GTC AAG AGC ATC (SEQ ID No 23)

K2 MUTANT OLIGO ATA GTC GCG AGC ATC (SEQ ID No 24)

WILD-TYPE SEQUENCE TCT TGG GGT ATC AAT (SEQ ID No 25)

R1 MUTANT OLIGO TCT TGG GCT ATC AAT (SEQ ID No 26)

After the mutant constructs were sequenced, these B3 domains were used to reconstitute the chimeric proteins consisting of residues 1-314 of BGP and residues 490-668 of CEA. These constructs have been shown to be positive for PR1A3 binding when transiently expressed in COS cells.

After insertion into pCDM8 and expression in COS cells, immunofluorescence experiments demonstrated that the constructs carrying the K2 (lysine 636 → alanine) and R3 (arginine 594 → alanine) mutations were no longer recognised by PR1A3 whereas the K1 (lysine 610 → alanine) mutation had no effect on binding (see Figure 10). These results implicate the residues K2 and R3 in the epitope recognised by PR1A3.

**Example 3: Preparation and properties of monoclonal antibodies reactive against CEA**

10

Monoclonal antibodies reactive against CEA were prepared by the method of Richman & Bodmer (1987).

*Tissues, cells, cell culture*

15

Fresh samples of normal large intestine and colorectal tumours were used. These were snap-frozen in liquid nitrogen and stored at -70°C. Frozen samples of extra-colonic normal human adult and fetal tissues were used.

20 The colorectal carcinoma cell lines used originated from different tumours. HT29 colon carcinoma cell line (Fogh and Trempe, 1975) was maintained in medium RPMI 1640 containing 10% fetal calf serum (FCS) and 37°C in 5% CO<sub>2</sub> in air at 100% humidity. LS174T, SW1222, SW48, SW620 and SW837 colorectal carcinoma cell lines (Tom *et al.*, 1976; Leibovitz *et al.*, 1976) were maintained in Dulbecco's modified Eagle's medium containing 10% FCS at 37°C in 10% CO<sub>2</sub> in air at 100% humidity.

P3/NS1/1-Ag-4-1 (NS1) is an 8-azaguanine-resistant BALB/c myeloma cell line. This was maintained in RPMI 1640 with 10% FCS and 2 x 10<sup>-5</sup>M 6-thioguanine.

30

Hybridomas produced in this study were initially cultured in RPMI 1640 with 20% FCS,  $10^{-4}$ M hypoxanthine,  $1.6 \times 10^{-5}$ M thymidine and  $10^{-5}$ M methotrexate (HAT). After cloning, hybridoma cells were weaned off HAT and maintained in RPMI 1640 with 10% FCS.

5

### *Immunizing materials*

BALB/c mice were immunized with 4 different preparations.

- 10 1. *Normal colorectal mucosal scrapings.* Samples of normal large intestine were pinned onto a cork board. After thorough rinsing (10 times) in cold, sterile phosphate-buffered saline-A, pH 7.4 (PBS-A), the mucosa was dissected from the muscularis mucosae by scraping with a scalpel. Mucosal scrapings were snap-frozen in liquid nitrogen and  
15 mechanically vibrated to a powder in a polypropylene vial containing a tungsten ballbearing. This material was emulsified in 0.2ml complete Freund's adjuvant and 0.2ml PBS-A. Animals received 0.2g wet tissue in 0.4ml emulsion per inoculation.
- 20 2. *Crude membrane preparations from normal colorectal epithelium.* Fresh normal colorectal mucosal scrapings were prepared as above. One gram of wet tissue was used for each membrane preparation. Tissue samples were thawed and Dounce-homogenized in 10ml sucrose buffer containing dithiothreitol (DTT) (250mM sucrose-RNase free: 50 mM  
25 triethanolamine-HCl pH 7.5; 60 mM  $MgCl_2$ ; 2mM DTT). Following centrifugation at 40,000g for 15 min, the nuclear and mitochondrial pellet was discarded. The supernatant was then centrifuged for a further 30 min at 20,000g. The microsomal pellet was retained and resuspended in 40% sucrose in 10mM Tris HCl pH 7.4. The sucrose solution was adjusted to  
30 obtain a refractometer reading of 1.392-5, overlaid with 25% sucrose in

10mm Tris HCl pH 7.4 (refractometer reading 1.375) and the sucrose gradient was centrifuged at 4°C for 15 hr at 65,000g. Membranes were recovered from the interface and washed twice in 10mm Tris pH 7.4; protein content was estimated by the method of Lowry *et al.* (1951). One  
5 gram of wet tissue yielded approximately 1mg of membrane protein. The membranes were suspended in PBS-A and complete Freund's adjuvant for injection. Animals received 0.4ml emulsion per inoculation.

3. *HT29 colon carcinoma cell line.* Animals received  $2 \times 10^6$  live  
10 trypsinized cells suspended in 0.4ml PBS-A per inoculation.

4. *Epitope as immunogen.* The immunogen is a cell carrying hybrid BGP-CEA B3-GPI protein or a cell transfected with CEA cDNA or cosmid. A mouse L cell transfected with CEA gene is used to immunise  
15 an appropriate mouse strain to give antibodies to CEA. Human tumour cells expressing CEA can also be used.

#### *Immunization and production of hybridomas*

20 Three fusions were carried out using spleens from BALB/c mice immunized by intraperitoneal inoculations according to the following protocol. In fusions 1 and 2, mice were immunized and boosted with mucosal scrapings and membrane preparations of normal colorectal epithelium (see "Immunizing materials" above). In fusion 3, initial  
25 immunization was with membrane preparations of normal colon and subsequent booster inoculations were with HT29 colon carcinoma cells. Animals received intraperitoneal injections of these materials 6 weeks, 2 weeks and 4 days prior to each fusion. In each case, the spleen was removed aseptically; a single-cell suspension was prepared mechanically  
30 and the spleen cells were fused with  $10^8$  NSI myeloma cells using 50%

polyethyleneglycol 4,000 (Merck) in RPMI 1640. The cells were plated into 24- or 96-well plates (Linbro, Flow, Irvine, Scotland) containing RPMI 1640 with HAT plus 20% FCS and mouse spleen cells as a feeder layer. The plates were incubated at 37°C in 5% CO<sub>2</sub> in air at 100% humidity. Hybridomas were generally visible microscopically at 14-21 days; initial screening to identify interesting colonies was performed prior to cloning. These colonies were cloned twice by picking single cells with a drawn-out Pasteur pipette, transferring them to individual wells of 96-well Microtitre plates containing mouse spleen cell feeders overlaid with 2ml RPMI 1640, HAT and 20% FCS, and cultured at 37°C in 5% CO<sub>2</sub> in air in 100% humidity.

*Screening assay for antibody production*

Screening for antibody production from all fusions was performed on tissue sections using an indirect immunoperoxidase technique. Cryostat sections (6µm thickness) were cut from snap-frozen cubes of normal large intestine. The sections were picked up on 10-well multitest slides (C.A. Hendley-Essex, England) precoated with 0.1% poly-l-lysine and allowed to dry in air for 30 min at room temperature. The sections were fixed in acetone for 15 min. Individual wells were incubated with 20µl unconcentrated hybridoma tissue culture supernatant for 30 min at room temperature in a humid chamber. Slides were washed twice in Tris-buffered saline (TBS) pH 7.6 (Tris, 605 mg, NaCl, 8g in 1 l distilled water) before incubation for 30 min at room temperature with peroxidase-conjugated rabbit anti-mouse immunoglobulin (DAKO, Copenhagen, Denmark) diluted 1:50 in TBS containing 5% normal human serum. The slides were washed again in TBS and then flooded with freshly prepared filtered solution of diaminobenzidine (Sigma, St. Louis, MO) 5mg in 10ml Tris HCl pH 7.6 containing 0.03% hydrogen peroxide. The peroxidase

substrate reaction was stopped after 5 min by washing in tap water and the slides were counterstained with Heyer's haematoxylin, dehydrated in alcohol and mounted in DPX (BDH, Poole, UK).

## 5 *Immunohistochemical methods*

1. *Indirect immunoperoxidase staining of formalin-fixed tissue.* To determine the reactivity of the MAbs with formalin-fixed, paraffin-embedded tissues, samples of normal large intestine were fixed in either  
10 (a) 10% neutral buffered formalin or (b) acid formalin (2% acetic acid in 10% formalin) for 2 hr. After routine processing 3-4 $\mu$ m sections were stained by the indirect peroxidase technique as described above (see "Screening"). Prior to staining, endogenous peroxidase activity was blocked by incubating the sections for 10 min in a humid chamber at room  
15 temperature with a freshly prepared solution of 0.5% hydrogen peroxide in methanol. After washing in tap water, slides were treated in one of 3 ways irrespective of the type of fixative used. (i) Stained directly; (ii) Digested in trypsin. For digestion slides were warmed at 37°C in distilled water and transferred to a freshly prepared solution of 0.1% trypsin  
20 (Sigma, type II), 0.1% CaCl<sub>2</sub>, pH 7.8 with NaOH for periods of 5-40 min (iii) Digested with other protease solutions. Warmed slides were transferred to a solution of protease (Sigma, type IV), 0.025% in TBS pH 7.6 for 5-15 min.

25 In (i) and (ii) above, enzyme reactions were stopped with cold running water. After thorough washing in water and TBS the digested sections were stained by the indirect immunoperoxidase technique.

2. *Indirect immunoperoxidase staining of frozen tissue sections.*  
30 Frozen sections of normal large intestine, other normal tissue and



- colorectal tumours were prepared as described above (see "Screening"). They were picked up on 4-well multitest slides (C.A. Hendly-Essex) and stained. They were not enzyme-digested and endogenous peroxidase activity was not blocked. All tissues were counterstained in Meyer's
- 5 haematoxylin, dehydrated in a graded alcohol series, cleared in xylene and mounted in DPX.
3. *Immunofluorescence of frozen sections.* Frozen sections of both normal colorectal tissue and some tumours were also examined by indirect
- 10 immunofluorescence. After fixing in acetone for 15 min, sections were washed in phosphate-buffered saline (PBS). Sections were then incubated with 20 $\mu$ l unconcentrated hybridoma supernatant for 30 min in a humid chamber, washed 3 times in PBS and incubated for a further 30 min with fluorescein-conjugated rabbit anti-mouse IgG (DAKO) diluted 1:40 in
- 15 PBS. After 3 further washes in PBS and a final wash in distilled water, the sections were mounted in Gelvato 20/30 (Monsanto, Springfield, MA) and viewed on a Leitz Orthoplan microscope with epifluorescence attachment.
- 20 4. *Immunocytochemical examination of cell lines.* Cells from the carcinoma lines were grown on glass slides. They were washed 3 times in PBS-A and then stained either live or after fixation in acetone for 10 min.
- 25 5. *Controls.* Immunohistochemical staining was controlled by the use of nonhybridoma tissue culture medium (RPMI 1640 with 10% FCS) as the primary layer. In addition, for immunoperoxidase staining the second antibody-enzyme conjugate and diaminobenzidine solution were also used individually. For immunofluorescence the FITC-conjugated
- 30 rabbit anti-mouse IgG was used alone. Non-specific staining by these

reagents was not observed.

*Assessment of colorectal tumours*

- 5 The colorectal adenocarcinomas were graded histologically by the criteria of Blenkinsopp *et al.* (1981) using sections stained with haematoxylin and eosin. For each antibody tumours were assessed as "negative" (no reactive cells), "heterogeneous" (some reactive cells) or "positive" (all cells reactive). Variations in the staining intensity between different cells  
10 of the same tumour or between tumour cells and the adjacent normal epithelium were sometimes seen but not quantitated.

Antibodies are screened by indirect immunofluorescent assays using CEA-positive cells air-dried and acetone-fixed on to cover slips. Also, whole  
15 cells or tissue sections carrying CEA are used and detection is by ELISA or radioimmunoassay (RIA).

Antibodies which are positive for CEA-expressing cell lines, negative for BGP- and NCA-expressing cell lines, and negative for BGP-CEA B3-  
20 expressing and BGP-CEA B3-BGPTM-expressing cell lines comprise antibodies of the invention.

**Example 4: Preparation of monospecific polyclonal antibodies reactive against CEA**

25

To prepare monospecific polyclonal antibodies reactive against CEA a suitable animal (rabbit, goat or the like) is immunized with hybrid BGP-CEA B3-GPI. The antisera so produced is then absorbed with purified BGP or cells expressing BGP to remove BGP-reactive antibodies and to  
30 leave the CEA reactive antibodies of the invention.

**Example 5: Radioimmunosintigraphy (RIS) of colorectal cancer**

Antibodies are used in RIS as described by Granowska *et al* (1989) in *Nuclear Medicine, trends and possibilities in nuclear medicine*, pp. 531-534 Schmidt & Buraggi (eds.), Schattauer, New York. A monoclonal antibody obtained by the method of Example 2 is labelled with indium-111 using the bifunctional chelate method of Hnatovich *et al* (1987). Imaging is undertaken using a Siemens 75 tube digitrac rotating gamma camera set with a medium energy parallel hole 'gallium' collimator and linked to a Nodecrest V77 computer. The camera is peaked to the two energies of In-111 with 15% and 20% windows and the counts are summed. Images are displayed on transparent film and in colour on the visual display unit of the computer.

Patients with primary or suspected recurrent colorectal cancer are selected by the surgeons and presented for RIS. The study is approved by the Administration of Radioactive Substances Advisory Committee of the Department of Health. Signed informed consent is obtained from each patient. Patients with a history of allergy to foreign proteins or with a positive skin test to the antibody are to be excluded. Patients with low rectal tumours were studied using multiple per rectal submucosal injection of antibody to undertake lymphoscintigraphy.

After the injection of 2-3mCi (80-120 MBq) of a known amount of activity, imaging was performed immediately, sometimes at 4 hours, at 24 hours with emission tomography, and at 48, 72 or 96 hours. Anterior and posterior views of the lower chest and upper abdomen, and lower abdomen and pelvis are obtained, together with images of six radioactive marker sources set on the bone land-marks to check repositioning of the patient and the image at each time point. Gamma camera images are also

made of the excised surgical specimen. The histological staging and grading of the tumour is undertaken. Specimens of the tumour, nearby mucosa and lymph nodes known to be involved or not involved with tumour are selected and counted, together with standards and appropriate background samples. Serial blood and urine samples are also assayed.

Blood clearance at 24 hours averages 51%; at 48 hours 33% and at 72 hours 27% of the injected dose taking the 5 minute sample volume as 100 per cent. Urine output is less than 3%.

10

Images of primary and recurrent colorectal cancer are of high quality. Tumour sites are clearly identified in the abdomen and pelvis often as early as 4 hours. Liver metastases are identified as focal defects on the early images which took up activity progressively with time. There is appreciably less normal bowel uptake than we are accustomed to with In-111 anti CEA (that is, anti-CEA antibodies that do not recognise the epitope recognised by PR1A3). Marrow and liver uptakes are similar. No false positive or false negative results are obtained. Single photon emission tomography is of no particular benefit since the planar images were so good.

20

Imaging of a surgical specimens shows that tumours and polyps have high uptake and that, unlike with other anti CEA antibodies (that do not recognise the epitope recognised by PR1A3) normal nodes are not visualised. The tumour to mucosa ratios are high ranging up to 47:1. Poorly differentiated tumours take up the antibody reasonably well and, on average, better than with In-111 anti CEA (that do not recognise the epitope recognised by PR1A3).

25

**Example 6: Humanising a mouse monoclonal antibody (CDR grafting)**

Complementary DNAs (cDNAs) encoding the variable regions of the monoclonal antibody were cloned and sequenced. Primers used for PCR  
5 cloning of the heavy chain V-region were from Orlandi *et al* (1989) and for the light chain V-region were from Jones & Bendig (1991). In each instance two sequences were given, one each for the parental NS1 light and heavy chains and unique sequences for a heavy chain and a light chain.

10

To confirm the specificity of the unique sequences they were expressed as a human-mouse chimaeric antibody where the mouse antibody V-regions were fused to human constant regions. The mouse antibody V<sub>H</sub>-region clone was linked to a cDNA clone of the C-regions of the human IgG<sub>1</sub>  
15 heavy chain NEWM (Kabat *et al* (1991) *supra*) by PCR techniques (see Figure 1). The mouse antibody V<sub>L</sub>-region was linked to a cDNA clone of the human kappa light chain REI (Kabat *et al* (1991) *supra*) by PCR techniques (see Figure 2).

20 The NEWM sequence is disclosed in Poljak *et al* (1977) *Biochemistry* **16**, 3412-3420 and the REI sequence is disclosed in Palm & Hilschmann (1973) *Z. Physiol. Chem.* **354**, 1651-1654 both incorporated herein by reference.

25 The chimaeric light and heavy chains were then inserted into the expression vector pCDM8 and the two plasmids co-transfected into COS cells. After eight days of culture antibody levels of approximately 1 µg/ml were determined in a human IgG Fc specific ELISA and the chimaeric antibody gave a positive immunofluorescent staining on MKN45 cells, a  
30 human gastric carcinoma cell line that carries the determinant identified

by PR1A3.

The DNA sequences of the V-regions were used to design humanised antibody. Analysis of the database allowed the selection of a human  
5 antibody with similarity to the mouse antibody (about 75% homology). This human antibody sequence was used as the template to design a humanised antibody sequence which was constructed from overlapping oligonucleotides and PCR and then linked to the cDNA of NEWM heavy chain.

10

The murine light chain had a homology of 70% to the human light chain. The light chain was then used as the template to construct humanised PR1A3 light chain using oligonucleotides and PCR by the method of Lewis & Crowe (1991).

15

## Methods

### *Heavy Chain* (see Figure 6)

20 Synthetic oligonucleotides, 1-6, code for the variable region of the heavy chain of the monoclonal antibody. These oligonucleotides (90mers) coded alternatively for the sense or the antisense strand of DNA, with 12 base pair overlaps between each sequential oligonucleotide. Primer dimer formation between pairs of oligonucleotides occurs, followed by PCR  
25 amplification.

The constant region was primed from a human heavy chain sequence contained within a plasmid. Incorporated into these primers was a 5' overlap with the 3' end of the variable region and a cloning site at the  
30 extreme 3' end of the gene.

PCR Conditions

- 95°C/1 min ← add *Taq* DNA polymerase
- 5    60°C/2 min    }  
      72°C/2 min    } x 30  
      95°C/1 min    }
- 60°C/2 min
- 72°C/7 min

10    *Light Chain* (see Figure 7)

The light chain was constructed in a similar manner to the heavy chain. Primers 1+2, 3+4, 5+6, 7+8 were PCR-amplified to produce overlapping fragments. The programme used was the same as for the heavy chain. The fragments were then joined using the following PCR programme.

Initially only the fragments are added.

- 20    93°C/1.5 min    }  
      37°C/1.0 min    } x 7  
      72°C/2.0 min    }

Outside primers are added.

- 25    93°C/1.5 min    }  
      37°C/1.0 min    } x 25  
      72°C/2.0 min    }

93°C/1.5 min

37°C/1.0 min

72°C/10.0 min

- 30    The template for this construct was a human light chain sequence contained within a plasmid. Primers (20-30mers) were designed to have a 3' region which was complementary to the human framework and a

foreign 5' region (either restriction enzyme sites or partial monoclonal antibody CDRs). The frameworks were amplified and the 5' foreign sequences were incorporated during this amplification. The individual fragments overlaps at the ends and were joined by overlapping PCR to  
5 form the complete gene.

Light and heavy chains have been inserted into the expression vector pCDM8 and antibody is being expressed in COS cells and its binding activity to MKN45 cells confirmed by immunofluorescence.

10

The V-regions of PR1A3 have been modelled using co-ordinates from structures of antibodies known from X-ray crystallographic studies. The complementarity determining regions (CDRs) were fitted to the framework structures using the canonical loop structures derived from Chothia *et al*  
15 (1992). Prominent features of the model include an additional residue, a tyrosine, in CDR3 of the light chain, and two glutamic acid residues, one in CDR2 of the heavy chain, and the other in CDR3 of the heavy chain. The two glutamic acids are unusual in that they are unpaired charges, but the additional tyrosine in CDR3-L causes this loop to kink and allows salt  
20 bridges to form between the two tyrosines in the CDR3-L loop and the unpaired glutamic acids of VH to stabilise the structure. The presence of these features is strongly suggestive that they are key to antigen recognition and that the epitope is positively charged.

25 Sequencing the V-region of the mouse PR1A3 heavy chains has consistently given a choice of two residues for the first amino acid of CDR1-H, both valine and glutamine have been found. Chimaeric antibodies of both isotypes are equally active in immunofluorescent studies with MKN45 cells and modelling allows both amino acids to be positioned  
30 with no constraints being imposed on the structure.



**Example 7: Acetone fixation of cells and determination of binding**

Cell suspensions, approximately  $10^5$  cells/ml in phosphate-buffered saline (PBS), were dropped onto microscope slides and allowed to dry, then  
5 immersed in acetone for 10 minutes and rinsed in PBS. Alternatively, coverslips were placed into a Petri dish containing culture medium such as RPMI 1640 containing 10% foetal calf serum, and cells seeded on to the coverslips. The Petri dishes were then incubated for between 48 and 72 hours at  $37^\circ\text{C}$ , and then were removed from the Petri dish, rinsed in  
10 PBS, immersed in acetone 10 minutes and then rinsed in PBS. The slides or coverslips were then incubated with the appropriate test antibody and washed. The test antibody either bound to or did not bind to the cell.

In order to detect binding of the test antibody, an anti-species antibody,  
15 labelled with fluorescein isothiocyanate (FITC), is added and then the cells washed. Binding is determined by measuring the fluorescence.

When mouse IgG is the test antibody FITC-conjugated sheep anti-mouse antibody (Sigma Chemical Co, Poole, Dorset, UK) is used as the probe.  
20

The cells used for binding studies are the colon carcinoma cell line HT-29 (ATCC HTB 38); COS-7 cells transfected with CEA cDNA; and COS-7 cells transfected with any of the DNA chimaeric constructs described in Example 1.  
25

Transfection of the COS-7 cells is by electroporation.

**Electroporation:** 200  $\mu\text{g}$  of plasmid DNA were mixed with 0.8 ml of cells in PBS, at a concentration of  $10^7$ - $10^8$  cells/ml. Cells were pulsed  
30 with 1 kv, 25  $\mu\text{FD}$  capacitance using a Bio-Rod Gene Pulser. Cells were

then placed on ice for at least 10 minutes before transfer to culture medium. Following overnight incubation at 37°C fresh medium was added to the cells.

5    **Example 8: Humanising a mouse monoclonal antibody (chimaeric fusions)**

10    The variable region of the murine monoclonal antibody was amplified by PCR using primers which added a *HindIII* restriction site to the 5' end of the sequence and the 3' end was designed to have a region of overlap with the 5' end of the constant domain of the kappa chain of the human antibody REI. This was amplified using standard amplification procedures (95°C for 1 minute followed by 30 cycles of 95°C for 1 minute, 60°C for 2 minutes and 72°C for 2 minutes with a final 72°C for 10 minutes). The  
15    REI kappa constant fragment was amplified under the same conditions, with the primers adding a 5' overlap with the 3' end of the monoclonal antibody kappa variable and a 3' *XbaI* site. These fragments were joined and extended by mutually primed synthesis to produce a sequence containing the variable region of the kappa chain of the murine  
20    monoclonal antibody and the constant domain from the kappa chain of the human antibody REI. PCR condition were 7 rounds of amplification, in a reaction containing both fragments, of 95°C for 2 minutes and 72°C for 4 minutes after which the outside primers were added and subjected to standard amplification procedures.

25

The heavy chain was constructed in a similar manner using the variable region of the heavy chain of the murine monoclonal antibody and the constant domains from the human heavy chain of NEWM.

30    These fragments were removed by restriction endonuclease digestion with

*HindIII* and *XbaI*. They were placed independently into the vectors pCDM8 and co-transfected into COS cells. The chimeric antibody was secreted into the medium and when tested by immunofluorescence against CEA expressed on the surface of MKN45 cells, exhibited all the characteristics of the construct which was murine in origin.

5

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CLAIMS

1. A molecule which (i) binds membrane-bound human carcinoembryonic antigen, (ii) binds a hybrid polypeptide consisting of  
 5 residues 1 to 314 of human biliary glycoprotein joined (N-C) to residues 490 to C-terminus of human carcino embryonic antigen, but (iii) does not bind to human biliary glycoprotein, but excluding an intact mouse monoclonal antibody comprising an IgG<sub>1</sub> group IIA heavy chain and a kappa group V light chain wherein the sequence of the V<sub>H</sub> chain is

10 QVKLQQSGPELKKPGETVKISCKASGYTFTVFGMNWVKQAPGKGLKWMGWINTKTGEATY  
 VEEFKGRFAFSLETSATTAYLQINNLIKNETAKYFCARWDFYDYVEAMDYWGQGT<sub>TTVS</sub>  
 S

, or wherein the sequence of the V<sub>H</sub> chain is as given immediately above  
 15 but the first amino acid residue of the V<sub>H</sub> CDR1 is glutamine

and in either case the sequence of the V<sub>L</sub> chain is

GDIVMTQSQRFMSTSVGDRVSVTCKASQNVGTNVAWYQQKPGQSPKALIYSASYRYS<sub>GV</sub>P  
 DRFTGSGSGTDFTLTISNVQSEDLAEYFCHQYYTYPLFTFGSGTKLEMKR.

20

2. A molecule according to Claim 1 which is an antibody comprising a human framework region and at least the complementarity determining regions of the V<sub>H</sub> chain and V<sub>L</sub> chain as defined in Claim 1 wherein for the V<sub>H</sub> chain CDR1 is VFGMN, CDR2 is WINTKTGEATYVEEFKG and  
 25 CDR3 is WDFYDYVEAMDY and for the V<sub>L</sub> chain CDR1 is KASQNVGTNVA, CDR2 is SASYRYS and CDR3 is HQYYTYPLFT.

3. A molecule according to Claim 2 wherein the antibody is a monoclonal antibody.

30

4. A molecule according to any one of the preceding claims further

comprising a directly or indirectly cytotoxic moiety.

5. A molecule according to any one of Claims 1 to 3 further comprising a readily-detectable label.

5

6. A molecule according to any one of Claims 1 to 5 for use in medicine.

7. Use of a molecule according to any one of Claims 1 to 3 and 5  
10 in the manufacture of a medicament for use in the diagnosis of colorectal carcinoma.

8. Use of a molecule according to any one of Claims 1 to 4 in the  
15 manufacture of a medicament for use in the treatment of colorectal carcinoma.

9. A process for making a monospecific antibody, the process  
comprising screening a pool of antibodies to select those monospecific  
antibodies which bind (i) human carcinoembryonic antigen, (ii) bind a  
20 hybrid polypeptide consisting of residues 1 to 314 of human biliary  
glycoprotein joined (N-C) to residues 490 to C-terminus of human  
carcinoembryonic antigen, but (iii) do not bind to human biliary  
glycoprotein.

25 10. A process according to Claim 9 wherein the monospecific  
antibody is a monoclonal antibody and the pool of antibodies is a pool of  
monoclonal antibodies.

11. A process according to Claim 9 wherein the antibodies within the  
30 pool comprise antibodies produced by recombinant DNA methods.

12. A process according to Claim 11 wherein the binding sites of the antibodies are displayed on the surface of a replicating vector.
13. A process according to Claim 12 wherein the replicating vector  
5 is a bacteriophage.
14. A monospecific antibody obtainable by the process of any one of Claims 9 to 13.
- 10 15. A hybrid polypeptide consisting of residues 1 to 314 of human biliary glycoprotein joined (N-C) to residues 490 to C-terminus of human carcinoembryonic antigen.
- 15 16. A hybrid polypeptide consisting of residues 1 to 314 of human biliary glycoprotein joined (N-C) to residues 490 to 643 of human carcinoembryonic antigen.
- 20 17. A hybrid polypeptide consisting of residues 1 to 314 of human biliary glycoprotein joined (N-C) to residues 490 to 644 of human carcinoembryonic antigen joined (N-C) to residues 391 to 430 of human biliary glycoprotein.
- 25 18. A hybrid polypeptide consisting of residues 1 to 314 of human biliary glycoprotein joined (N-C) to residues 490 to 642 of human carcinoembryonic antigen joined (N-C) to residues 387 to 430 of human biliary glycoprotein.

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	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	
RF-TS3 backbone	Q	V	Q	L	V	Q	S	G	S	E	L	K	K	P	G	A	S	V	K	
Humanised Heavy	Q	V	Q	L	V	Q	S	G	S	E	L	K	K	P	G	A	S	V	K	
Murine Heavy	Q	V	K	L	Q	Q	S	G	P	E	L	K	K	P	G	E	T	V	K	
	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35				
RF-TS3 backbone	V	S	C	K	A	S	G	Y	T	F	T	S	Y	A	M	N				
Humanised Heavy	V	S	C	K	A	S	G	Y	T	F	T	V	F	G	M	N				
Murine Heavy	I	S	C	K	A	S	G	Y	T	F	T	V	F	G	M	N				
	36	37	38	39	40	41	42	43	44	45	46	47	48	49						
RF-TS3 backbone	W	V	R	Q	A	P	G	Q	G	L	E	W	M	G						
Humanised Heavy	W	V	R	Q	A	P	G	Q	G	L	E	W	M	G						
Murine Heavy	W	V	K	Q	A	P	G	K	G	L	K	W	M	G						
	50	51	52	52a	53	54	55	56	57	58	59	60	61	62	63	64	65			
RF-TS3 backbone	W	I	N	T	N	T	G	N	P	T	Y	A	Q	G	F	T	G			
Humanised Heavy	W	I	N	T	K	T	G	E	A	T	Y	V	E	E	F	K	G			
Murine Heavy	W	I	N	T	K	T	G	E	A	T	Y	V	E	E	F	K	G			
	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	a	b	c
RF-TS3 backbone	R	F	V	F	S	L	D	T	S	V	S	T	A	Y	L	Q	I	S	S	L
Humanised Heavy	R	F	V	F	S	L	D	T	S	V	S	T	A	Y	L	Q	I	S	S	L
Murine Heavy	R	F	A	F	S	L	E	T	S	A	T	T	A	Y	L	Q	I	N	N	L
	83	84	85	86	87	88	89	90	91	92	93	94								
RF-TS3 backbone	K	A	D	D	T	A	V	Y	Y	C	A	R								
Humanised Heavy	K	A	D	D	T	A	V	Y	Y	C	A	R								
Murine Heavy	K	N	E	D	T	A	K	Y	F	C	A	R								
	95	96	97	98	99	100	a	b	c	k	101									
RF-TS3 backbone	E	D	S	N	G	Y	L	I	-	F	D									
Humanised Heavy	W	D	F	Y	D	Y	V	E	A	M	D									
Murine Heavy	W	D	F	Y	D	Y	V	E	A	M	D									
	102	103	104	105	106	107	108	109	110	111	112	113								
RF-TS3 backbone	Y	W	D	Q	G	T	L	V	I	V	S	S								
Humanised Heavy	Y	W	G	Q	G	T	T	V	T	V	S	S								
Murine Heavy	Y	W	G	Q	G	T	T	V	T	V	S	S								

Figure 1



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REI backbone	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Humanized Kappa	D	I	Q	M	T	Q	S	P	S	S	L	S	A	S	V
Murine Kappa	D	I	Q	M	T	Q	S	P	S	S	L	S	A	S	V
	D	I	V	M	T	Q	S	Q	R	F	M	S	T	S	V
REI backbone	16	17	18	19	20	21	22	23	24	25	26	27	28	29	
Humanized Kappa	G	D	R	V	T	I	T	C	Q	A	S	Q	D	I	
Murine Kappa	G	D	R	V	T	I	T	C	K	A	S	Q	N	V	
	G	D	R	V	S	V	T	C	K	A	S	Q	N	V	
REI backbone	30	31	32	33	34	35	36	37	38	39	40	41	42	43	
Humanized Kappa	I	K	Y	L	A	W	Y	Q	Q	T	P	G	K	A	
Murine Kappa	G	T	N	V	A	W	Y	Q	Q	K	P	G	K	A	
	G	T	N	V	A	W	Y	Q	Q	K	P	G	Q	S	
REI backbone	44	45	46	47	48	49	50	51	52	53	54	55	56		
Humanized Kappa	P	K	L	L	I	Y	E	A	S	N	L	Q	A		
Murine Kappa	P	K	L	L	I	Y	S	A	S	Y	R	Y	S		
	P	K	A	L	I	Y	S	A	S	Y	R	Y	S		
REI backbone	57	58	59	60	61	62	63	64	65	66	67	68	69	70	71
Humanized Kappa	G	V	P	S	R	F	S	G	S	G	S	G	T	D	Y
Murine Kappa	G	V	P	S	R	F	S	G	S	G	S	G	T	D	F
	G	V	P	D	R	F	T	G	S	G	S	G	T	D	F
REI backbone	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86
Humanized Kappa	T	F	T	I	S	S	L	Q	P	E	D	I	A	T	Y
Murine Kappa	T	F	T	I	S	S	L	Q	P	E	D	I	A	T	Y
	T	L	T	I	S	N	V	Q	S	E	D	L	A	E	Y
REI backbone	87	88	89	90	91	92	93	94	95	95a	96	97			
Humanized Kappa	Y	C	Q	Q	Y	Q	S	L	P	-	Y	T			
Murine Kappa	Y	C	H	Q	Y	Y	T	Y	P	L	F	T			
	F	C	H	Q	Y	Y	T	Y	P	L	F	T			
REI backbone	98	99	100	101	102	103	104	105	106	107	108				
Humanized Kappa	F	G	Q	G	T	K	I	E	I	T	R				
Murine Kappa	F	G	Q	G	T	K	V	E	I	K	R				
	F	G	S	G	T	K	L	E	M	K	R				

Figure 2

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CEA leader	-34	MESPSAPPHRWCI PWQRLLLTASLLTFFWNPPTTA	-1
NCA leader		.GP.L...C.LHV..KEV.....	
CEA N-terminus	1	KLTIESTPFNVAEGKEVLLLVHNLPHQLHFGYSWKGERVDGNRQIIGYVIGTQQATPGPA	
		.....A.....NRI.....SL.V.....	
		YSGREIIYPNASLLIQNIIONDTGFYTLHVIKSDLVNEEATGQFRVYP	108
		.....T.....VT.....Q.....H...	
CEA repeat 1	109	ELPKPSISSNNSKPVEDKDAVAFCEPETQDATYLVWVNNQSLPVSRLQLSNGNRTLT	
CEA repeat 2	287	.P...F.T...N...E...L...I.NT.....D.....	
CEA repeat 3	465	.....A.NT.....G.....M...	
NCA repeat		.....N.....NT.....G.....	
		FNVTRNDTASYKCEQNPVSARRSDSVILNVLYGPDAPTISPNTSYRSGENLNLSCHAA	
		LS.....VGP.E.GI..EL.VDH..P.....D.....SY.Y..P.V..S.....	
		....ARA.V.GI..S...N...P.T.D.....T.I...PDS..L..A.....S.	
		LS.K...AG..E..I...A..N...P.T.....G.....SKAN..P.....	
		SNPPAQYSWFVNGTFQQSTQELFIPNITVNNSGSVTCQAHNSDTGLNRTTITVYA	286
		.....LID.NI..H.....S...EK...L.....N..AS.HS.....K....S. 464	
		..SP.....RI..IP..H..V...AK..P..N.T.A.FVS.LA..R.NSI.KS....S. 642	
		.....IT.....M.....A.....M.....SG	
CEA C-terminus	643	SGTSPGLSAGATVGIMIGVLGVGVALI	668
NCA C-terminus		--SA.V...V.....T....AR....	

Figure 3

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FIGURE 4 (page 1 of 2)

27 54  
 ATG GGG CAC CTC TCA GCC CCA CTT CAC AGA GTG CGT GTA CCC TGG CAG GGG CTT  
 MET Gly His Leu Ser Ala Pro Leu His Arg Val Arg Val Pro Trp Gln Gly Leu

81 108  
 CTG CTC ACA GCC TCA CTT CTA ACC TTC TGG AAC CCG CCC ACC ACT GCC CAG CTC  
 Leu Leu Thr Ala Ser Leu Leu Thr Phe Trp Asn Pro Pro Thr Thr Ala Gln Leu  
 1

135 162  
 ACT ACT GAA TCC ATG CCA TTC AAT GTT GCA GAG GGG AAG GAG GTT CTT CTC CTT  
 Thr Thr Glu Ser MET Pro Phe Asn Val Ala Glu Gly Lys Glu Val Leu Leu Leu  
 16

189 216  
 GTC CAC AAT CTG CCC CAG CAA CTT TTT GGC TAC AGC TGG TAC AAA GGG GAA AGA  
 Val His Asn Leu Pro Gln Gln Leu Phe Gly Tyr Ser Trp Tyr Lys Gly Glu Arg

243 270  
 GTG GAT GGC AAC CGT CAA ATT GTA GGA TAT GCA ATA GGA ACT CAA CAA GCT ACC  
 Val Asp Gly Asn Arg Gln Ile Val Gly Tyr Ala Ile Gly Thr Gln Gln Ala Thr

297 324  
 CCA GGG CCC GCA AAC AGC GGT CGA GAG ACA ATA TAC CCC AAT GCA TCC CTG CTG  
 Pro Gly Pro Ala Asn Ser Gly Arg Glu Thr Ile Tyr Pro Asn Ala Ser Leu Leu  
 66

351 378  
 ATC CAG AAC GTC ACC CAG AAT GAC ACA GGA TTC TAC ACC CTA CAA GTC ATA AAG  
 Ile Gln Asn Val Thr Gln Asn Asp Thr Gly Phe Tyr Thr Leu Gln Val Ile Lys

405 432  
 TCA GAT CTT GTG AAT GAA GAA GCA ACT GGA CAG TTC CAT GTA TAC CCG GAG CTG  
 Ser Asp Leu Val Asn Glu Glu Ala Thr Gly Gln Phe His Val Tyr Pro Glu Leu

459 486  
 CCC AAG CCC TCC ATC TCC AGC AAC AAC TCC AAC CCT GTG GAG GAC AAG GAT GCT  
 Pro Lys Pro Ser Ile Ser Ser Asn Asn Ser Asn Pro Val Glu Asp Lys Asp Ala  
 116

513 540  
 GTG GCC TTC ACC TGT GAA CCT GAG ACT CAG GAC ACA ACC TAC CTG TGG TGG ATA  
 Val Ala Phe Thr Cys Glu Pro Glu Thr Gln Asp Thr Thr Tyr Leu Trp Trp Ile

567 594  
 AAC AAT CAG AGC CTC CCG GTC AGT CCC AGG CTG CAG CTG TCC AAT GGC AAC AGG  
 Asn Asn Gln Ser Leu Pro Val Ser Pro Arg Leu Gln Leu Ser Asn Gly Asn Arg

621 648  
 ACC CTC ACT CTA CTC AGT GTC ACA AGG AAT GAC ACA GGA CCC TAT GAG TGT GAA  
 Thr Leu Thr Leu Leu Ser Val Thr Arg Asn Asp Thr Gly Pro Tyr Glu Cys Glu  
 166

675 702  
 ATA CAG AAC CCA GTG AGT GCG AAC CGC AGT GAC CCA GTC ACC TTG AAT GTC ACC  
 Ile Gln Asn Pro Val Ser Ala Asn Arg Ser Asp Pro Val Thr Leu Asn Val Thr

729 756  
 TAT GGC CCG GAC ACC CCC ACC ATT TCC CCT TCA GAC ACC TAT TAC CGT CCA GGG  
 Tyr Gly Pro Asp Thr Pro Thr Ile Ser Pro Ser Asp Thr Tyr Tyr Arg Pro Gly  
 216

783 810  
 GCA AAC CTC AGC CTC TCC TGC TAT GCA GCC TCT AAC CCA CCT GCA CAG TAC TCC  
 Ala Asn Leu Ser Leu Ser Cys Tyr Ala Ala Ser Asn Pro Pro Ala Gln Tyr Ser

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FIGURE 4 (page 2 of 2)

837 864  
 TGG CTT ATC AAT GGA ACA TTC CAG CAA AGC ACA CAA GAG CTC TTT ATC CCT AAC  
 Trp Leu Ile Asn Gly Thr Phe Gln Gln Ser Thr Gln Glu Leu Phe Ile Pro Asn

891 918  
 ATC ACT GTG AAT AAT AGT GGA TCC TAT ACC TGC CAC GCC AAT AAC TCA GTC ACT  
 Ile Thr Val Asn Asn Ser Gly Ser Tyr Thr Cys His Ala Asn Asn Ser Val Thr  
 266

945 972  
 GGC TGC AAC AGG ACC ACA GTC AAG ACG ATC ATA GTC ACT GAG CTA AGT CCA GTA  
 Gly Cys Asn Arg Thr Thr Val Lys Thr Ile Ile Val Thr Glu Leu Ser Pro Val

999 1026  
 GTA GCA AAG CCC CAA ATC AAA GCC AAG ACC ACA GTC ACA GGA GAT AAG GAC  
 Val Ala Lys Pro Gln Ile Lys Ala Ser Lys Thr Thr Val Thr Gly Asp Lys Asp

1053 1080  
 TCT GTG AAC CTG ACC TGC TCC ACA AAT GAC ACT GGA ATC TCC ATC CGT TGG TTC  
 Ser Val Asn Leu Thr Cys Ser Thr Asn Asp Thr Gly Ile Ser Ile Arg Trp Phe  
 316

1107 1134  
 TTC AAA AAC CAG AGT CTC CCG TCC TCG GAG AGG ATG AAG CTG TCC CAG GGC AAC  
 Phe Lys Asn Gln Ser Leu Pro Ser Ser Glu Arg MET Lys Leu Ser Gln Gly Asn

1161 1188  
 ACC ACC CTC AGC ATA AAC CCT GTC AAG AGG GAG GAT GCT GGG ACG TAT TGG TGT  
 Thr Thr Leu Ser Ile Asn Pro Val Lys Arg Glu Asp Ala Gly Thr Tyr Trp Cys

1215 1242  
 GAG GTC TTC AAC CCA ATC AGT AAG AAC CAA AGC GAC CCC ATC ATG CTG AAC GTA  
 Glu Val Phe Asn Pro Ile Ser Lys Asn Gln Ser Asp Pro Ile MET Leu Asn Val  
 366

1269 1296  
 AAC TAT AAT GCT CTA CCA CAA GAA AAT GGC CTC TCA CCT GGG GCC ATT GCT GGC  
 Asn Tyr Asn Ala Leu Pro Gln Glu Asn Gly Leu Ser Pro Gly Ala Ile Ala Gly

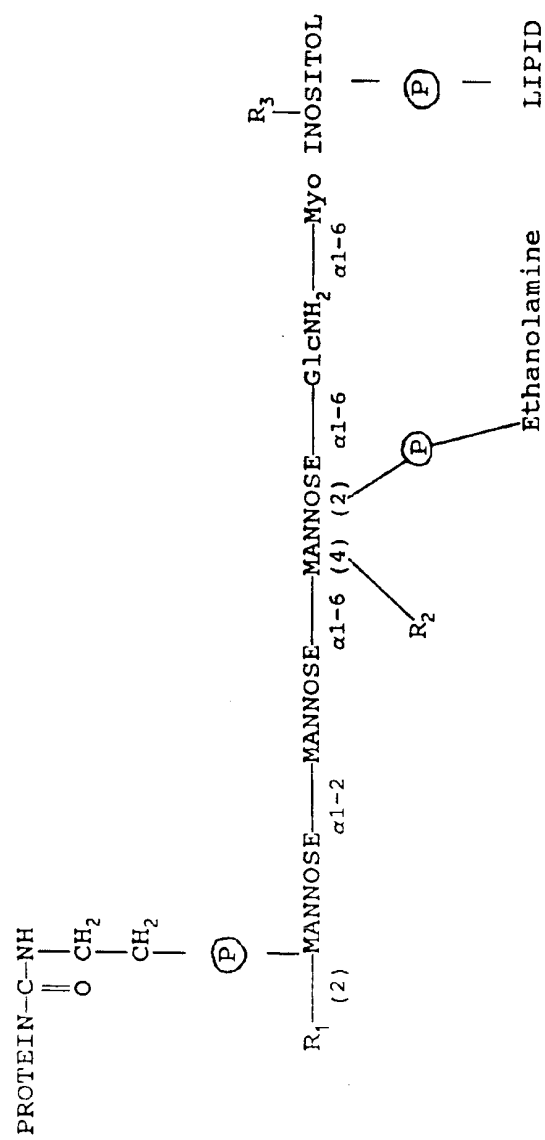
1323 1350  
 ATT GTG ATT GGA GTA GTG GCC CTG GTT GCT CTG ATA GCA GTA GCC CTG GCA TGT  
 Ile Val Ile Gly Val Val Ala Leu Val Ala Leu Ile Ala Val Ala Leu Ala Cys  
 416

1377 1404  
 TTT CTG CAT TTC GGG AAG ACC GGC AGC TCA GGA CCA CTC CAA TGA CCC ACC TAA  
 Phe Leu His Phe Gly Lys Thr Gly Ser Ser Gly Pro Leu Gln  
 430

1431 1458  
 CAA GAT GAA TGA AGT TAC TTA TCT ACC CTG AAC TTT GAA GCC CAG CAA CCC ACA

CAA CCA ACT TCA CTT

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R<sub>1</sub> = OH pr  $\alpha$  MANNOSE  
 R<sub>2</sub> = OH or  $\beta$  GalNAC  
 R<sub>3</sub> = OH or PALMITATE

Figure 5

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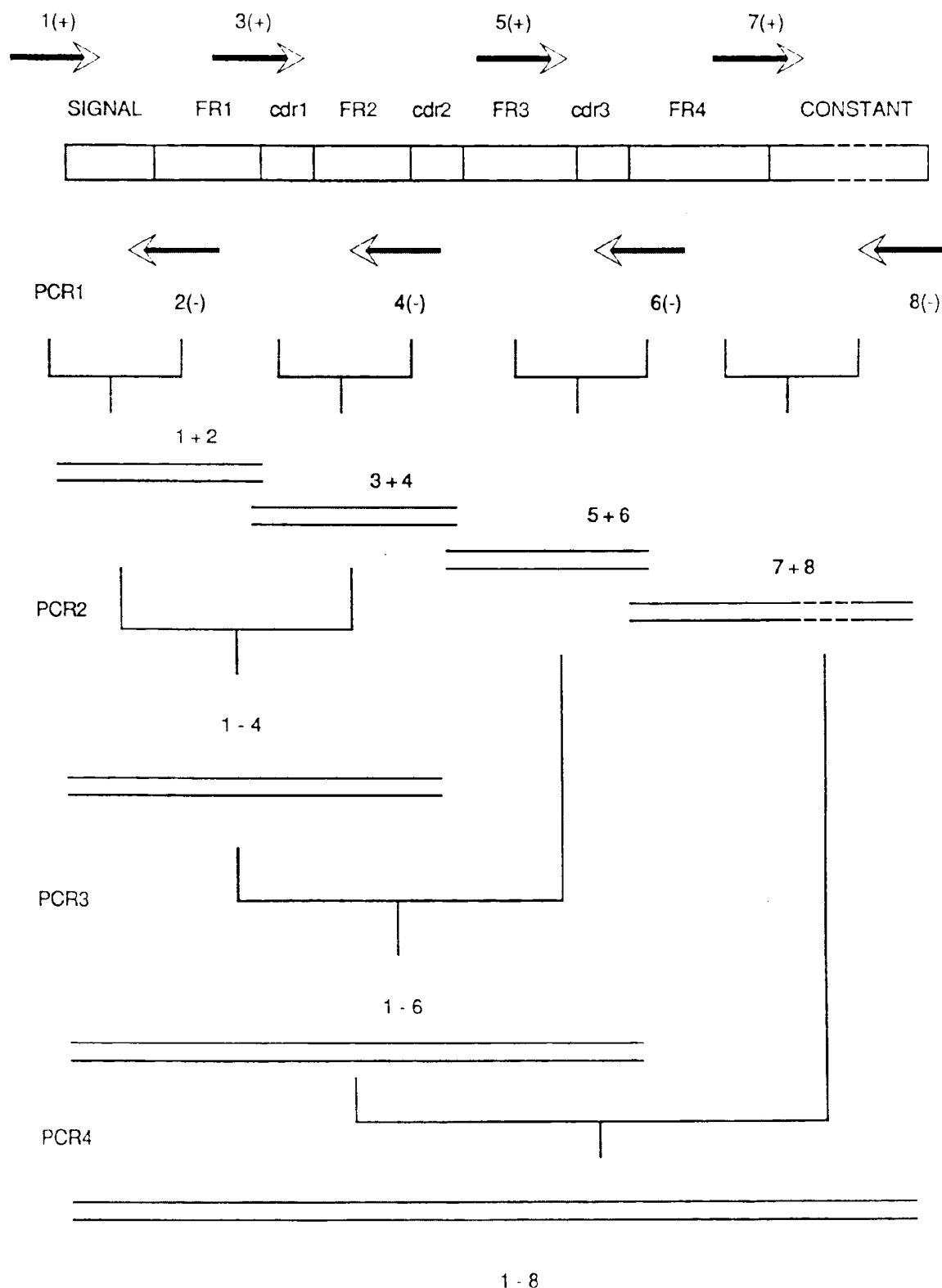


Figure 6

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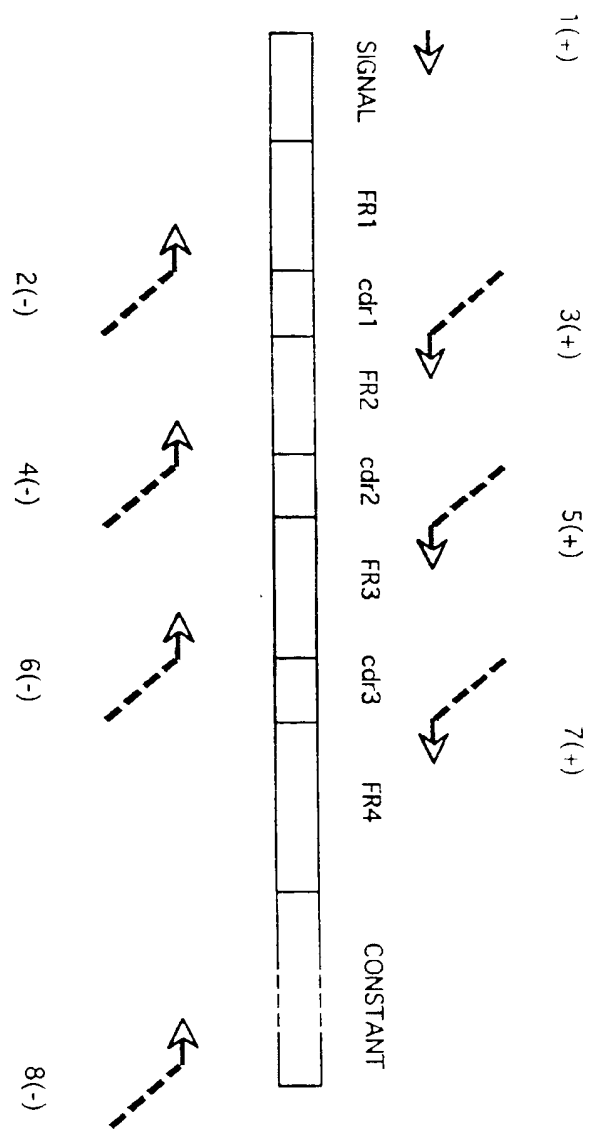


Figure 7

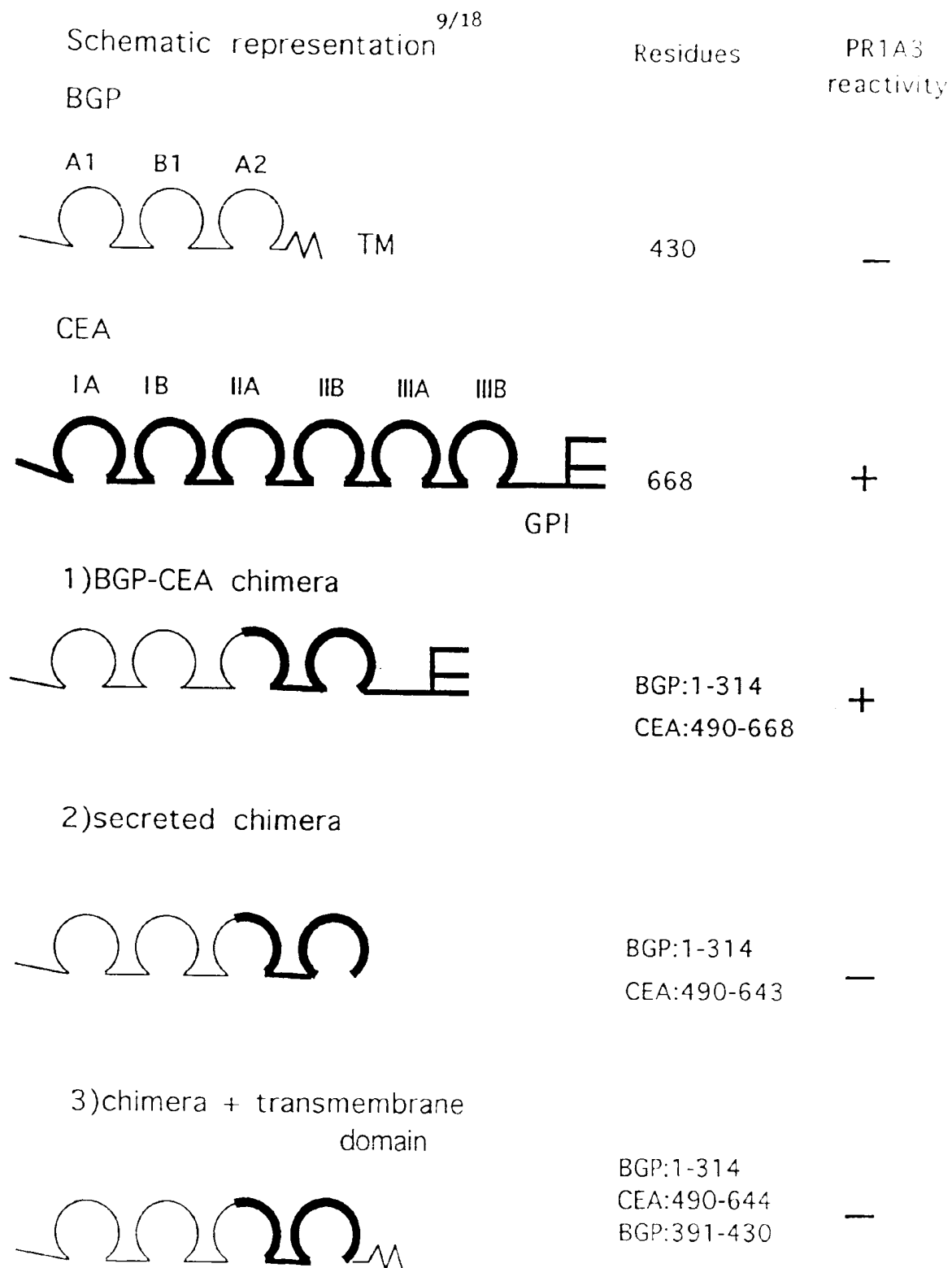


Figure 8



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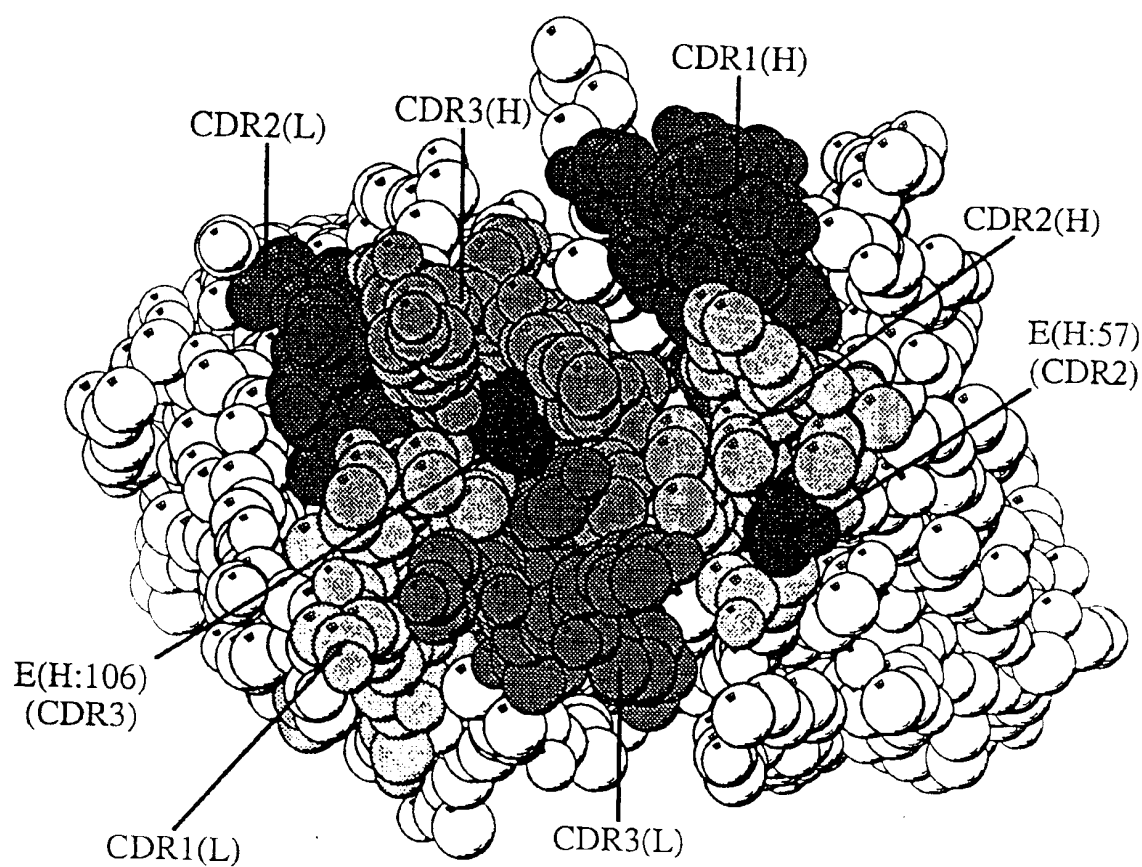


Figure 9

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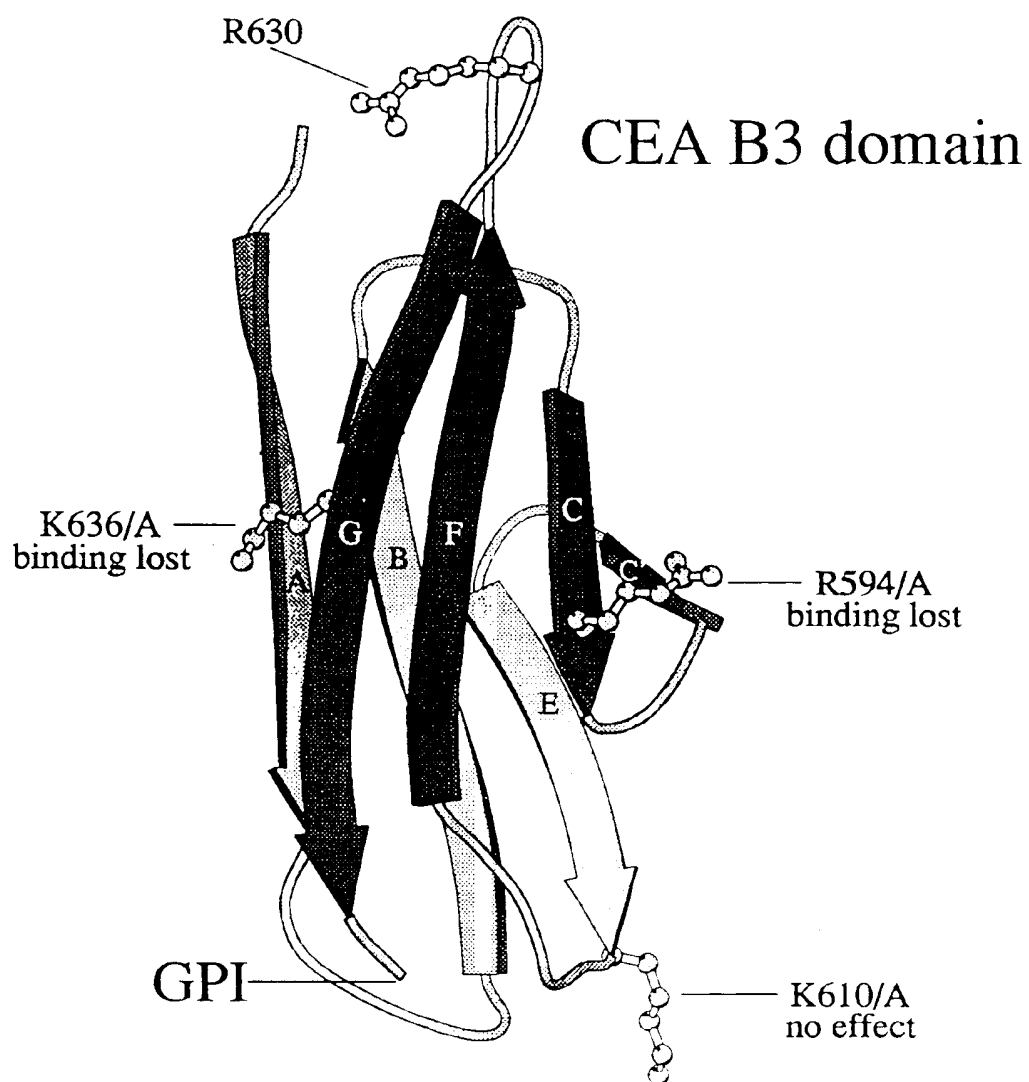


Figure 10

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FIGURE 11 (page 1 of 2)

ATG GGA CCC CCC TCA GCC CCT CCC TGC AGA TTG CAT GTC CCC TGG AAG GAG GTC	27	54
MET Gly Pro Pro Ser Ala Pro Pro Cys Arg Leu His Val Pro Trp Lys Glu Val		
CTG CTC ACA GCC TCA CTT CTA ACC TTC TGG AAC CCA CCC ACC ACT GCC AAG CTC	81	108
Leu Leu Thr Ala Ser Leu Leu Thr Phe Trp Asn Pro Pro Thr Thr Ala Lys Leu		
ACT ATT GAA TCC ACG CCA TTC AAT GTC GCA GAG GGG AAG GAG GTT CTT CTA CTC	135	162
Thr Ile Glu Ser Thr Pro Phe Asn Val Ala Glu Gly Lys Glu Val Leu Leu Leu		
GCC CAC AAC CTG CCC CAG AAT CGT ATT GGT TAC AGC TGG TAC AAA GCG GAA AGA	189	216
Ala His Asn Leu Pro Gln Asn Arg Ile Gly Tyr Ser Trp Tyr Lys Ala Glu Arg		
GTG GAT GGC AAC AGT CTA ATT GTA GGA TAT GTA ATA GGA ACT CAA CAA GCT ACC	243	270
Val Asp Gly Asn Ser Leu Ile Val Gly Tyr Val Ile Gly Thr Gln Gln Ala Thr		
CCA GGG CCC GCA TAC AGT GGT CGA GAG ACA ATA TAC CCC AAT GCA TCC CTG CTG	297	324
Pro Gly Pro Ala Tyr Ser Gly Arg Glu Thr Ile Tyr Pro Asn Ala Ser Leu Leu		
ATC CAG AAC GTC ACC CAG ATT GAC ACA GGA TTC TAT ACC CTA CAA GTC ATA AAG	351	378
Ile Gln Asn Val Thr Gln Ile Asp Thr Gly Phe Tyr Thr Leu Gln Val Ile Lys		
TCA GAT CTT CTC AAT GAA GAA GCA ACC GGA CAG TTC CAT GTA TAC CCG GAG CTG	405	432
Ser Asp Leu Val Asn Glu Glu Ala Thr Gly Gln Phe His Val Tyr Pro Glu Leu		
CCC AAG CCC TCC ATC TCC AGC AAC AAC TCC AAC CCC GTG GAG GAC AAG GAT GCT	459	486
Pro Lys Pro Ser Ile Ser Ser Asn Asn Ser Asn Pro Val Glu Asp Lys Asp Ala		
GTG GCC TTC ACC TGT GAA CCT GAG GTT CAG AAC ACA ACC TAC GTC TGG TGG GTA	513	540
Val Ala Phe Thr Cys Glu Pro Glu Val Gln Asn Thr Thr Tyr Val Trp Trp Val		
AAT GGT CAG AGC CTC CCG GTC AGT CCC AGG CTG CAG CTC TCC AAT GGC AAC ATG	567	594
Asn Gly Gln Ser Leu Pro Val Ser Pro Arg Leu Gln Leu Ser Asn Gly Asn MET		
ACC CTC ACT CTA CTC AGC GTC AAA AGG AAC GAT GCA GGA TCC TAT GAA TGT GAA	621	648
Thr Leu Thr Leu Leu Ser Val Lys Arg Asn Asp Ala Gly Ser Tyr Glu Cys Glu		
ATA CAG AAC CCA GCG AGT GCC AAC CGC AGT GAC CCA GTC ACC CTG AAT GTC CTC	675	702
Ile Gln Asn Pro Ala Ser Ala Asn Arg Ser Asp Pro Val Thr Leu Asn Val Leu		
TAT GGC CCA GAT GGC CCC ACC ATT TCC CCC TCA AAG CCC AAT TAC CGT CCA GGG	729	756
Tyr Gly Pro Asp Gly Pro Thr Ile Ser Pro Ser Lys Ala Asn Tyr Arg Pro Gly		

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FIGURE 11 (page 2 of 2)

783 810  
 GAA AAT CTG AAC CTC TCC TCG CAC GCA GCC TCT AAC CCA CCT GCA CAG TAC TCT  
 Glu Asn Leu Asn Leu Ser Ser His Ala Ala Ser Asn Pro Pro Ala Gln Tyr Ser

837 864  
 TGG TTT ATC AAT GGG ACC TTC CAG CAA TCC ACA CAA GAG CTC TTT ATC CCC AAC  
 Trp Phe Ile Asn Gly Thr Phe Gln Gln Ser Thr Gln Glu Leu Phe Ile Pro Asn

891 918  
 ATC ACT GTG AAT AAT AGC GGA TCC TAT ATG TGC CAA GCC CAT AAC TCA GCC ACT  
 Ile Thr Val Asn Asn Ser Gly Ser Tyr MET Cys Gln Ala His Asn Ser Ala Thr

945 972  
 GGC CTC AAT AGG ACC ACA GTC ACG ATG ATC ACA GTC TCT GGA AGT GCT CCT GTC  
 Gly Leu Asn Arg Thr Thr Val Thr MET Ile Thr Val Ser Gly Ser Ala Pro Val

999 1026  
 CTC TCA GCT GTG GCC ACC GTC GGC ATC ACG ATT GGA GTG CTG GCC AGG GTC GCT  
 Leu Ser Ala Val Ala Thr Val Gly Ile Thr Ile Gly Val Leu Ala Arg Val Ala

1053 1080  
 CTG ATA TAG CAG CCC TGG TGT ATT TTC GAT ATT TCA GGA AGA CTG GCA GAT TGG  
 Leu Ile .

ACC AGA CCC TGA ATT CTT CTA GC

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FIGURE 12 (page 1 of 3)

27 54  
TCG GCC CCT CCC CAC ACA TGG TGC ATC CCC TGG CAG AGG CTC CTG CTC ACA GCC  
Ser Ala Pro Pro His Arg Trp Cys Ile Pro Trp Gln Arg Leu Leu Leu Thr Ala

81 108  
TCA CTT CTA ACC TTC TGG AAC CCG CCC ACC ACT GCC AAG CTC ACT ATT GAA TCC  
Ser Leu Leu Thr Phe Trp Asn Pro Pro Thr Thr Ala Lys Leu Thr Ile Glu Ser  
1

135 162  
ACG CCG TTC AAT GTC GCA GAG GGG AAG GAG GTG CTT CTA CTT GTC CAC AAT CTG  
Thr Pro Phe Asn Val Ala Glu Gly Lys Glu Val Leu Leu Leu Val His Asn Leu

189 216  
CCC CAG CAT CTT TTT GGC TAC AGC TGG TAC AAA GGT GAA AGA GTG GAT GGC AAC  
Pro Gln His Leu Phe Gly Tyr Ser Trp Tyr Lys Gly Glu Arg Val Asp Gly Asn

243 270  
CGT CAA ATT ATA GGA TAT CTA ATA GGA ACT CAA CAA GCT ACC CCA GGG CCC GCA  
Arg Gln Ile Ile Gly Tyr Val Ile Gly Thr Gln Gln Ala Thr Pro Gly Pro Ala

297 324  
TAC AGT GGT CGA GAG ATA ATA TAC CCC AAT GCA TCC CTG CTG ATC CAG AAC ATC  
Tyr Ser Gly Arg Glu Ile Ile Tyr Pro Asn Ala Ser Leu Leu Ile Gln Asn Ile

351 378  
ATC CAG AAT GAC ACA GGA TTC TAC ACC CTA CAC GTC ATA AAG TCA GAT CTT GTG  
Ile Gln Asn Asp Thr Gly Phe Tyr Thr Leu His Val Ile Lys Ser Asp Leu Val

405 432  
AAT GAA GAA GCA ACT GGC CAG TTC CGG GTA TAC CCG GAG CTG CCC AAG CCC TCC  
Asn Glu Glu Ala Thr Gly Gln Phe Arg Val Tyr Pro Glu Leu Pro Lys Pro Ser  
108 109

459 486  
ATC TCC AGC AAC AAC TCC AAA CCC GTG GAG GAC AAG GAT GCT GTG GCC TTC ACC  
Ile Ser Ser Asn Asn Ser Lys Pro Val Glu Asp Lys Asp Ala Val Ala Phe Thr

513 540  
TGT GAA CCT GAG ACT CAG GAC GCA ACC TAC CTG TGG TGG GTA AAC AAT CAG AGC  
Cys Glu Pro Glu Thr Gln Asp Ala Thr Tyr Leu Trp Trp Val Asn Asn Gln Ser

567 594  
CTC CCG GTC AGT CCC AGG CTG CAG CTG TCC AAT GGC AAC AGG ACC CTC ACT CTA  
Leu Pro Val Ser Pro Arg Leu Gln Leu Ser Asn Gly Asn Arg Thr Leu Thr Leu

621 648  
TTC AAT GTC ACA AGA AAT GAC ACA GCA AGC TAC AAA TGT GAA ACC CAG AAC CCA  
Phe Asn Val Thr Arg Asn Asp Thr Ala Ser Tyr Lys Cys Glu Thr Gln Asn Pro

675 702  
GTG AGT GCC AGG CGC AGT GAT TCA GTC ATC CTG AAT GTC CTC TAT GGC CCG GAT  
Val Ser Ala Arg Arg Ser Asp Ser Val Ile Leu Asn Val Leu Tyr Gly Pro Asp

729 756  
GCC CCC ACC ATT TCC CCT CTA AAC ACA TCT TAC AGA TCA GGG GAA AAT CTG AAC  
Ala Pro Thr Ile Ser Pro Leu Asn Thr Ser Tyr Arg Ser Gly Glu Asn Leu Asn

783 810  
CTC TCC TGC CAC GCA GCC TCT AAC CCA CCT GCA CAG TAC TCT TGG TTT GTC AAT  
Leu Ser Cys His Ala Ala Ser Asn Pro Pro Ala Gln Tyr Ser Trp Phe Val Asn

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FIGURE 12 (page 2 of 3)

837 864  
 GGG ACT TTC CAG CAA TCC ACC CAA GAG CTC TTT ATC CCC AAC ATC ACT GTG AAT  
 Gly Thr Phe Gln Gln Ser Thr Gln Glu Leu Phe Ile Pro Asn Ile Thr Val Asn

891 918  
 AAT AGT GGA TCC TAT ACG TGC CAA GCC CAT AAC TCA GAC ACT GGC CTC AAT AGG  
 Asn Ser Gly Ser Tyr Thr Cys Gln Ala His Asn Ser Asp Thr Gly Leu Asn Arg

945 972  
 ACC ACA GTC ACG ACG ATC ACA GTC TAT GCA GAG CCA CCC AAA CCC TTC ATC ACC  
 Thr Thr Val Thr Thr Ile Thr Val Tyr Ala Glu Pro Pro Lys Pro Phe Ile Thr  
 286 287

999 1026  
 AGC AAC AAC TCC AAC CCC GTG GAG GAT GAG GAT GCT GTA GCC TTA ACC TGT GAA  
 Ser Asn Asn Ser Asn Pro Val Glu Asp Glu Asp Ala Val Ala Leu Thr Cys Glu

1053 1080  
 CCT GAG ATT CAG AAC ACA ACC TAC CTG TGG TGG GTA AAT AAT CAG ACC CTC CCG  
 Pro Glu Ile Gln Asn Thr Thr Tyr Leu Trp Trp Val Asn Asn Gln Ser Leu Pro

1107 1134  
 GTC AGT CCC AGG CTG CAG CTG TCC AAT GAC AAC AGG ACC CTC ACT CTA CTC AGT  
 Val Ser Pro Arg Leu Gln Leu Ser Asn Asp Asn Arg Thr Leu Thr Leu Leu Ser

1161 1188  
 GTC ACA AGG AAT GAT GTA GGA CCC TAT GAG TGT GGA ATC CAG AAC GAA TTA AGT  
 Val Thr Arg Asn Asp Val Gly Pro Tyr Glu Cys Gly Ile Gln Asn Glu Leu Ser

1215 1242  
 GTT GAC CAC AGC GAC CCA GTC ATC CTG AAT GTC CTC TAT GGC CCA GAC GAC CCC  
 Val Asp His Ser Asp Pro Val Ile Leu Asn Val Leu Tyr Gly Pro Asp Asp Pro

1269 1296  
 ACC ATT TCC CCC TCA TAC ACC TAT TAC CGT CCA GGG GTG AAC CTC AGC CTC TCC  
 Thr Ile Ser Pro Ser Tyr Thr Tyr Tyr Arg Pro Gly Val Asn Leu Ser Leu Ser

1323 1350  
 TGC CAT GCA GCC TCT AAC CCA CCT GCA CAG TAT TCT TGG CTG ATT GAT GGG AAC  
 Cys His Ala Ala Ser Asn Pro Pro Ala Gln Tyr Ser Trp Leu Ile Asp Gly Asn

1377 1404  
 ATC CAG CAA CAC ACA CAA GAG CTC TTT ATC TCC AAC ATC ACT GAG AAG AAC AGC  
 Ile Gln Gln His Thr Gln Glu Leu Phe Ile Ser Asn Ile Thr Glu Lys Asn Ser

1431 1458  
 GGA CTC TAT ACC TGC CAG GCC AAT AAC TCA GCC AGT GGC CAC AGC AGG ACT ACA  
 Gly Leu Tyr Thr Cys Gln Ala Asn Asn Ser Ala Ser Gly His Ser Arg Thr Thr

1485 1512  
 GTC AAG ACA ATC ACA GTC TCT GCG GAG CTG CCC AAG CCC TCC ATC TCC AGC AAC  
 Val Lys Thr Ile Thr Val Ser Ala Glu Leu Pro Lys Pro Ser Ile Ser Ser Asn  
 464 465

1539 1566  
 AAC TCC AAA CCC GTG GAG GAC AAG GAT GCT GTG GCC TTC ACC TGT GAA CCT GAG  
 Asn Ser Lys Pro Val Glu Asp Lys Asp Ala Val Ala Phe Thr Cys Glu Pro Glu

1593 1620  
 GCT CAG AAC ACA ACC TAC CTG TGG TGG GTA AAT GGT CAG AGC CTC CCA GTC AGT  
 Ala Gln Asn Thr Thr Tyr Leu Trp Trp Val Asn Gly Gln Ser Leu Pro Val Ser

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FIGURE 12 (page 3 of 3)

1647 1674  
 CCC AGG CTG CAG CTG TCC AAT GGC AAC AGG ACC CTC ACT CTA TTC AAT GTC ACA  
 Pro Arg Leu Gln Leu Ser Asn Gly Asn Arg Thr Leu Thr Leu Phe Asn Val Thr

1701 1728  
 AGA AAT GAC GCA AGA GCC TAT GTA TGT GGA ATC CAG AAC TCA GTG AGT GCA AAC  
 Arg Asn Asp Ala Arg Ala Tyr Val Cys Gly Ile Gln Asn Ser Val Ser Ala Asn

1755 1782  
 CGC AGT GAC CCA GTC ACC CTG GAT GTC CTC TAT GGG CCG GAC ACC CCC ATC ATT  
 Arg Ser Asp Pro Val Thr Leu Asp Val Leu Tyr Gly Pro Asp Thr Pro Ile Ile

1809 1836  
 TCC CCC CCA GAC TCG TCT TAC CTT TCG GGA GCG AAC CTC AAC CTC TCC TGC CAC  
 Ser Pro Pro Asp Ser Ser Tyr Leu Ser Gly Ala Asn Leu Asn Leu Ser Cys His

1863 1890  
 TCG GCC TCT AAC CCA TCC CCG CAG TAT TCT TGG CGT ATC AAT GGG ATA CCG CAG  
 Ser Ala Ser Asn Pro Ser Pro Gln Tyr Ser Trp Arg Ile Asn Gly Ile Pro Gln

1917 1944  
 CAA CAC ACA CAA GTT CTC TTT ATC GCC AAA ATC ACG CCA AAT AAT AAC GGG ACC  
 Gln His Thr Gln Val Leu Phe Ile Ala Lys Ile Thr Pro Asn Asn Asn Gly Thr

1971 1998  
 TAT GCC TGT TTT GTC TCT AAC TTG GCT ACT GGC CGC AAT AAT TCC ATA GTC AAG  
 Tyr Ala Cys Phe Val Ser Asn Leu Ala Thr Gly Arg Asn Asn Ser Ile Val Lys

2025 2052  
 AGC ATC ACA GTC TCT GCA TCT GGA ACT TCT CCT GGT CTC TCA GCT GGG GCC ACT  
 Ser Ile Thr Val Ser Ala Ser Gly Thr Ser Pro Gly Leu Ser Ala Gly Ala Thr  
 642 643

2079  
 GTC GGC ATC ATG ATT GGA GTC CTG GTT GGG GTT GCT CTG ATA TAG  
 Val Gly Ile MET Ile Gly Val Leu Val Gly Val Ala Leu Ile  
 668

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FIGURE 13

27 54  
ATG GGC ATC AAG ATG GAG TCA CAT TCC CTG GTC TTT GTA TAC ATG TTG CTG TGG  
MET Gly Ile Lys MET Glu Ser His Ser Leu Val Phe Val Tyr MET Leu Leu Trp

81 108  
TTG TCT GGT GTT GAT GGA GAC ATT GTG ATG ACC CAG TCT CAA AGA TTC ATG TCC  
Leu Ser Gly Val Asp Gly Asp Ile Val MET Thr Gln Ser Gln Arg Phe MET Ser

135 162  
ACA TCA GTA GGA GAC AGG GTC AGC GTC ACC TGC AAG GCC AGT CAG AAT GTG GGT  
Thr Ser Val Gly Asp Arg Val Ser Val Thr Cys Lys Ala Ser Gln Asn Val Gly

189 216  
ACT AAT GTT GCC TGG TAT CAA CAG AAA CCA GGA CAA TCC CCT AAA GCA CTG ATT  
Thr Asn Val Ala Trp Tyr Gln Gln Lys Pro Gly Gln Ser Pro Lys Ala Leu Ile

243 270  
TAC TCG GCA TCC TAC CCG TAC AGT GGA GTC CCT GAT CGC TTC ACA GGC AGT GGA  
Tyr Ser Ala Ser Tyr Arg Tyr Ser Gly Val Pro Asp Arg Phe Thr Gly Ser Gly

297 324  
TCT GGG ACA GAT TTC ACT CTC ACC ATC AGC AAT GTA CAG TCT GAA GAC TTG GCG  
Ser Gly Thr Asp Phe Thr Leu Thr Ile Ser Asn Val Gln Ser Glu Asp Leu Ala

351 378  
GAG TAT TTC TGT CAC CAA TAT TAC ACC TAT CCT CTA TTC ACG TTC GGC TCG GGG  
Glu Tyr Phe Cys His Gln Tyr Tyr Thr Tyr Pro Leu Phe Thr Phe Gly Ser Gly

ACA AAG TTG GAA ATG AAA  
Thr Lys Leu Glu MET Lys



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FIGURE 14

27 54  
 ATG GGA TGG AGC TGT ATC ATG CTC TTC TTG GCA GCA ACA GCT ACA GGT GTC CAC  
 MET Gly Trp Ser Cys Ile MET Leu Phe Leu Ala Ala Thr Ala Gly Val His

81 108  
 TCC CAG GTG AAG CTG CAG CAG TCA GGA CCT GAG TTG AAG AAG CCT GGA GAG ACA  
 Ser Gln Val Lys Leu Gln Gln Ser Gly Pro Glu Leu Lys Lys Pro Gly Glu Thr

135 162  
 GTC AAG ATC TCC TGC AAG GCT TCT GGA TAT ACC TTC ACA GTG TTT GGA ATG AAC  
 Val Lys Ile Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Val Phe Gly MET Asn

189 216  
 TGG GTG AAG CAG GCT CCT GGA AAG GGT TTA AAG TGG ATG GGC TGG ATA AAC ACC  
 Trp Val Lys Gln Ala Pro Gly Lys Gly Leu Lys Trp MET Gly Trp Ile Asn Thr

243 270  
 AAA ACT GGA GAG GCA ACA TAT GTT GAA GAG TTT AAG GGA CGG TTT GCC TTC TCT  
 Lys Thr Gly Glu Ala Thr Tyr Val Glu Glu Phe Lys Gly Arg Phe Ala Phe Ser

297 324  
 TTG GAG ACC TCT GCC ACC ACT GCC TAT TTG CAG ATC AAC AAC CTC AAA AAT GAG  
 Leu Glu Thr Ser Ala Thr Thr Ala Tyr Leu Gln Ile Asn Asn Leu Lys Asn Glu

351 378  
 GAC ACG GCT AAA TAT TTC TGT GCA AGA TGG GAC TTC TAT GAT TAC GTG GAG GCT  
 Asp Thr Ala Lys Tyr Phe Cys Ala Arg Trp Asp Phe Tyr Asp Tyr Val Glu Ala

405  
 ATG GAC TAC TGG GGC CAA GGG ACC ACG GTC ACC GTC TCC  
 MET Asp Tyr Trp Gly Gln Gly Thr Thr Val Thr Val Ser

## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/GB 94/01816

A. CLASSIFICATION OF SUBJECT MATTER		
IPC 6	C07K16/30 C07K19/00	C12P21/08 A61K47/48 A61K39/395 G01N33/577
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED		
Minimum documentation searched (classification system followed by classification symbols)		
IPC 6 C07K C12P A61K G01N		
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched		
Electronic data base consulted during the international search (name of data base and, where practical, search terms used)		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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A	INTERNATIONAL JOURNAL OF CANCER, vol.39, no.3, 15 March 1987, GENEVA, SWITZERLAND pages 317 - 328 P. RICHMAN ET AL. 'Monoclonal antibodies to human colorectal epithelium: Markers for differentiation and tumour characterization.' cited in the application see the whole document --- -/--	1,4-10, 14
<input checked="" type="checkbox"/> Further documents are listed in the continuation of box C. <input checked="" type="checkbox"/> Patent family members are listed in annex.		
* Special categories of cited documents : 'A' document defining the general state of the art which is not considered to be of particular relevance 'E' earlier document but published on or after the international filing date 'L' document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) 'O' document referring to an oral disclosure, use, exhibition or other means 'P' document published prior to the international filing date but later than the priority date claimed 'T' later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention 'X' document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone 'Y' document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art. '&' document member of the same patent family		
Date of the actual completion of the international search		Date of mailing of the international search report
2 November 1994		22 -11- 1994
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+ 31-70) 340-2040, Tx. 31 651 epo nl, Fax (+ 31-70) 340-3016		Authorized officer  Nooij, F

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## INTERNATIONAL SEARCH REPORT

International application No.  
PCT/GB 94/01816

C(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
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A	AMERICAN JOURNAL OF CLINICAL PATHOLOGY, vol.94, no.2, August 1990, PHILADELPHIA PA, USA pages 157 - 164 K. SHEAHAN ET AL. 'Differential reactivities of carcinoembryonic antigen (CEA) and CEA-related monoclonal and polyclonal antibodies in common epithelial malignancies.' see the whole document ---	1,4-10, 14
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Information on patent family members

International application No.

PCT/GB 94/01816

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		DE-A- 3879452	22-04-93
		ES-T- 2053810	01-08-94
		JP-A- 2154696	14-06-90
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